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Obesity is characterized by chronic, low-grade inflammation that leads to insulin resistance and metabolic syndrome. It is well accepted that the expansion of adipose tissue leads to a state of relative hypoxia that is associated with inflammation and adipocyte dysfunction. While numerous studies have established hypoxia-inducible factor-1α (HIF-1α) as a critical transcription factor for metabolic adaptation during low oxygen conditions, less is known about the regulation and functionality of HIF-1α induced by inflammation under normoxic conditions. Data presented in this dissertation demonstrated that HIF-1α is induced by the inflammatory cytokine Oncostatin M (OSM) in 3T3-L1 adipocytes during normoxia. The induction of HIF-1α by OSM was regulated at the level of transcription and dependent on ERK and AKT signaling pathways. Furthermore, data show that HIF-1α was responsible for metabolic and vasculature adaptations, such as increased glycolytic gene expression, glucose uptake, lactate production and angiogenic gene expression induced by OSM. We further demonstrated that OSM contributes to adipose tissue remodeling by reducing cell growth and inhibiting adipogenesis. Specifically, OSM inhibited differentiation during mitotic clonal expansion (MCE) and prevented the induction of peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), adipocyte protein 2 (aP2) and adiponectin (ADPN). As MCE is characterized by several rounds of cell cycle, examination of cyclins revealed that OSM dramatically reduced cyclin D1. Furthermore, HIF-1α knockdown partially restored PPARγ, C/EBPα, aP2 and cyclin D1, suggesting that HIF-1α is a mediator of the inhibition of cell growth and differentiation by OSM. Data

also show that the addition of hypoxia-mimetic, $CoCl_2$ and OSM led to a synergistic effect on HIF-1α, which suggests that the hypoxia and inflammation implicated during obesity may magnify the effect on downstream targets of HIF-1α. These data demonstrate an essential role for HIF-1α in the effect of OSM on adaptations in adipocytes during normoxia. Collectively, the findings presented here provide insight into the molecular mechanisms by which hypoxia and inflammation contribute to metabolic adaptation and adipose tissue remodeling.

ROLE FOR HYPOXIA-INDUCIBLE FACTOR-1α IN ONCOSTATIN-INDUCED ADAPTATIONS IN ADIPOCYTES DURING NORMOXIA

by

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Approved by

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Dedicated to my fellow women scientists. The future is female.

APPROVAL PAGE

This dissertation, written by Dana M. DeSilva, has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Significance of Research

According to the World Health Organization (WHO), the prevalence of obesity worldwide tripled between 1975 and 2016 and over 340 million of these individuals classified as overweight or obese were children and adolescents. This ever-expanding obesity epidemic is a serious public health concern due to the obesity-related complications such as cardiovascular disease, diabetes, and cancer, as well as the increased health care costs associated with these diseases. Although organizations such as WHO and the Centers for Disease Control and Prevention (CDC) have initiated projects and funding that aim to reduce obesity, the prevalence of obesity and diabetes remains alarmingly high, signifying the need for improved therapeutic strategies that target these obesity-related diseases. It is well established that obesity is associated with chronic, low-grade inflammation, which is directly linked to insulin resistance and metabolic syndrome. Furthermore, it has been determined that a link between inflammation and low oxygen tension exists and contributes to adipocyte dysfunction. Overwhelming evidence suggests that the expansion of adipose tissue leads to a state of a hypoxia, or low oxygen tension, and that the effects are mediated through hypoxia-inducible factor-1α (HIF-1α). Although numerous studies have investigated the relationship between inflammation and hypoxia, the regulation and effects of HIF-1α induced by inflammatory cytokines remain largely unknown. Collectively, the proposed research is significant, as it advances our understanding of the regulation of HIF-1α by

inflammatory cytokines and establishes a functional role for HIF-1α in adipocyte dysfunction, which could lead to the creation of new therapeutic targets for improving the deleterious effects of hypoxia and inflammation implicated in obesity.

Review of Literature

Obesity. Before the 20th century, humans in both developed and underdeveloped countries struggled with poverty, food scarcity and malnutrition as a result of low economic productivity and development. A major concern continued to be the number of underweight individuals, as this affected both work efficiency and survival. However, as countries became more industrialized, the number of adults with excess weight began to outnumber those who were underweight (1). In fact, increasing industrialization radically transformed the food system, allowing for an increase of processed food, which is commonly energy dense and highly refined (2). This change in the food system has proven to be a critical turning point and major driver of the global obesity epidemic (2,3).

Obesity is characterized by the expansion of adipose tissue and is most commonly defined by body mass index (BMI). A BMI measurement between 25-30 kg/m^2 is considered overweight, while BMI over 30 kg/m^2 is classified as obese (1). In 2014, 36.5% of U.S. adults and 17% of U.S. youth were classified as obese (4). In 2013, the prevalence of obese adults was 31% and the prevalence of overweight or obese was 65% in developed countries. In developing countries, the prevalence of obesity was lower, at about 11%, but had similar trends in overweight and obesity as compared to developed countries. Between 1980-2013, the prevalence of overweight and obesity worldwide increased by 28% for adults and 47% for children (5).

This global rise in obesity has major health consequences. Among the many complications that arise from obesity including liver disease, cancer, and neurodegeneration, perhaps the most concerning is metabolic syndrome (3,6). Metabolic syndrome is defined as a conglomeration of factors, such as insulin resistance, chronic low-grade inflammation and dyslipidemia that all directly increase the risk of cardiovascular disease and type 2 diabetes (7). According to the CDC, in 2015, the prevalence of diabetes in the U.S. population of individuals' ages 18 or older was 12.2%, or 30.2 million adults. Of these adults, 7.2 million were unaware or did not report that they had the disease. Historically, children with diabetes were diagnosed with type 1 diabetes. However, with the increase in childhood obesity, the CDC reported that in 2015, the annual increase for type 2 diabetes in children within the U.S. was 4.8%, and from 2011-2012 5,300 children were newly diagnosed with this disease (8). Furthermore, according to the International Diabetes Federation, 415 million people worldwide were affected by diabetes and it is predicted to rise to 642 million by 2040 (9). The health care costs associated with obesity related complications in the U.S. alone are almost $2,000 per year for each obese individual, which amounts to $150 billion annually (10).

These statistics demonstrate that obesity and its related complications have severe health and financial consequences for the U.S. and the world at large. Due to the many factors that contribute to obesity, finding ways to prevent, reverse, and manage obesity related diseases, is critical for the health of future generations. Increased research and understanding of cellular mechanisms of adipocyte dysfunction implicated during obesity could lead to the improvement of the effects of obesity-related disorders.

Adipose Tissue. Adipose tissue is a complex organ composed of a variety of cell types and is located throughout the body (11,12). While the primary cell type of

adipose tissue is the adipocyte, including preadipocytes and adipocytes, adipose tissue is also comprised of macrophages, fibroblasts, lymphocytes, and endothelial cells (13–15). Adipose tissue has a remarkable capacity for expansion, which allows for energy storage and mobilization under fasting or starvation conditions (11,16). This is a particularly fascinating evolutionary feature as it has allowed humans to survive in severe food shortages and times of uncertainty (17). It also serves as thermal insulation for the body and as cushion for organ protection (15).

While adipose tissue was once considered to function mainly as a storage depot for triglycerides, we now understand that it is a highly active metabolic organ, able to secrete a variety of bioactive compounds such as adipokines that play a critical role in energy homeostasis, as well as vasculogenesis and immunity (11–14). It was in the early 50's that this view of adipose tissue as merely storage began to shift when G.C. Kennedy came up with what became known as the lipostatic hypothesis. Kennedy proposed that the adipose tissue had a feedback signal to regulate energy expenditure and food intake (18). This hypothesis became heavily explored and confirmed. In 1973 researchers found that genetically obese mice were unable to produce a sufficient satiety factor to regulate food consumption; diabetic mice were able to produce the satiety factor but could not respond to it (19).

Finally, in 1994, the obese gene was identified and sequenced, which allowed the lipostatic signal derived from this gene to become known as leptin (20). The discovery of leptin was of great significance not only for its ability to regulate body fat, but because this showed that adipose tissue is an endocrine organ and that there is a physiologic system that regulates metabolism. This new recognition of adipose tissue as an endocrine organ led to the discovery of many proteins and factors that are secreted

from adipose tissue, termed adipokines (21). It is now widely accepted that adipose tissue is a dynamic organ, critical for health and disease and plays huge roles in metabolic regulation, obesity, inflammation and insulin resistance. Overnutrition leads to obese adipose tissue and dysregulated adipokine secretion, which contributes to obesity-related diseases such as diabetes, hyperlipidemia and hypertension (22).

Adipose Tissue Dysfunction. As individuals gain weight, adipocytes within the tissue increase in number (hyperplasia) and size (hypertrophy). These mechanisms, along with infiltration of macrophages, fibrosis and reduced extracellular matrix flexibility encompass the term adipose tissue remodeling (23,24). Hypertrophy is considered the most critical mechanism for obese adipose tissue (25). It is this expansion of adipocytes that leads to decreased insulin sensitivity, reduced adiponectin secretion and increased pro-inflammatory cytokines and hypoxia (25,26). Enlarged adipocyte size is associated with type 2 diabetes (27). Several mechanisms for this correlation include impaired differentiation, increased release of free fatty acids (FFAs), increased secretion of cytokines and a reduction in adiponectin, which typically functions to increase insulin sensitivity (27,28).

It is now well established that obesity is associated with chronic, low-grade inflammation. It is this state of inflammation that is accompanied by increased secretion of pro-inflammatory and pro-atherogenic cytokines, which characterizes the term adipocyte dysfunction (28). These cytokines contribute greatly to insulin resistance and type 2 diabetes. For example, tumor necrosis factor alpha (TNF-α) increases lipolysis, which promotes the release of fatty acids into circulation and leads to insulin resistance in the skeletal muscle and liver (29,30). Adipocyte hypertrophy is also associated with an increased number of macrophages, which contributes to the pro-inflammatory phenotype

of obese adipose tissue (25). In short, adipose tissue expansion and adipocyte hypertrophy, such as found in obesity, is associated with increased inflammation, impaired adipogenesis, dysregulated glucose and lipid metabolism, altered adipokine secretion and hypoxia (25,31).

Hypoxia in Adipose Tissue, HIF-1α. Oxygen is an essential requirement for all aerobic organisms. While not typically considered a major macronutrient, oxygen is required for respiration and other key metabolic processes. For example, the conversion of fatty acids and glucose to ATP via oxidative phosphorylation requires an ample supply of oxygen (32). The general level of tissue oxygenation in humans is between 40 and 50 mmHg (33). However, as illustrated in Fig. 1.1, as adipose tissue expands, the amount of oxygen to the tissue decreases, causing a state of hypoxia, or low oxygen tension (33,34). In fact, during obesity, the postprandial adipose tissue blood flow response is attenuated and the underlying mechanism is associated with insulin sensitivity (35,36). In mammals, cells sense this low extracellular oxygen tension and evolutionarily responses are activated.

Hypoxia, such as implicated in obesity, leads to adipose tissue dysfunction by causing several functional changes. First, it causes a switch from oxidative metabolism to anaerobic metabolism by inhibiting the expression of genes associated with oxidation and increasing glycolytic gene expression (Fig.1.2) (37,38). This metabolic adaptation causes increased glucose uptake and lactate production (39). Additionally, one proposed underlying mechanism for the inflammation found in obesity is hypoxia. Hypoxia causes an increase in several inflammatory adipokines such as interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), plasminogen activator inhibitor 1 (PAI-1) and causes a reduction in anti-inflammatory molecules such as adiponectin (39,40).

Other effects of hypoxia that contribute to adipocyte dysfunction include modulation of lipid metabolism and reduced insulin sensitivity (41).

The primary transcriptional signals in response to hypoxia are the members of the HIF family (33,42). The three main hypoxia-inducible factors (HIFS) are HIF-1, HIF-2, and HIF-3. While the role of HIF-3 in hypoxia is much less understood than HIF-1 and HIF-2, it is HIF-1 that has become known as the 'master regulator of oxygen homeostasis' (33,43,44). HIF-1 is a heterodimeric complex composed of HIF-1α and HIF-1β, also known as aryl hydrocarbon nuclear translocator (ARNT), that is present in all metazoan species (45,46). Although HIF-1β is constitutively expressed, HIF-1α has a very short half-life and is continually synthesized and degraded under normoxia (33,42,44). Within the HIF-1α subunit, there are three hydroxylation sites; two prolyl residues in the oxygen-dependent degradation domain (ODDD) and one asparaginyl residue in the C-terminal transactivation domain (C-TAD). In well-oxygenated environments, Fe (II), oxygen and prolyl hydroxylase domain-containing enzymes (PHDs) catalyze prolyl hydroxylation. Proline residue hydroxylation allows HIF-1α to be recognized and targeted for degradation by von Hippel-Lindau protein (pVHL), which leads to ubiquitination and proteasomeal degradation (33,47). In the absence of oxygen, during hypoxic stress, PHD activity is inhibited, the pVHL cannot bind and HIF-1α becomes stabilized. HIF-1α then translocates to the nucleus, heterodimerizes with HIF-1β and allows for transcription of target genes by binding to hypoxia-response elements (HREs) in the regulatory regions of these genes (44,46).

While there are several mechanisms by which hypoxia induces molecular and cellular responses, many of the hypoxic effects are mediated specifically through HIF-1α. This allows researchers to study HIF-1α dependent genes under normoxic

conditions by using chemical hypoxia mimetics such as $CoCl_2$, which inhibits PHD activity and thereby stabilizes the protein. HIF-1α transcriptionally regulates many different genes that encode for proteins involved in a variety of processes such as angiogenesis, inflammation, cellular proliferation/survival and glucose metabolism (33,48,49).

Hypoxia, Oxidative Metabolism, Glucose Utilization and Lactate Production. The primary mechanism for creating an adequate supply of cellular ATP is mitochondrial respiration. This process is dependent on oxygen as the final electron acceptor in the electron transport chain (ETC). The transfer of electrons through the various ETC complexes creates a gradient that drives the synthesis of ATP. If too little oxygen is present, the flow of electrons is halted, leading to a reduced oxidative metabolism and increased production of reactive oxygen species (ROS) (50). As illustrated in Fig.1.2, a critical metabolic adaptation to hypoxia is the stabilization of HIF-1α, which causes a switch from oxidative to glycolytic metabolism (33,44,45). HIF-1α is able to maintain ATP production through increased anaerobic metabolism and reduce uncontrolled toxic ROS generated by the ETC (44).

Pyruvate dehydrogenase kinase 1 (PDK1) phosphorylates pyruvate dehydrogenase (PDH), which inactivates the complex that converts pyruvate to acetyl-CoA for entry into the TCA cycle. During hypoxia, HIF-1α stimulates PDK1 activity, resulting in a decreased flux through the TCA cycle and inhibition of oxidative phosphorylation (51). In fact, under hypoxic conditions, HIF-1α null mouse embryo fibroblasts (MEFs) fail to activate PDK1, leading to increased ROS and apoptosis. Additionally, HIF-1α regulates cytochrome c oxidase 4 (COX4) in the ETC by activating COX4-2, which optimizes the efficiency of respiration during hypoxia (52). These HIF-1α

dependent adaptations are important for the prevention of toxic ROS and conservation of ATP (44,51).

Reduced oxidative phosphorylation during hypoxia causes a shift towards anaerobic metabolism through increasing glucose transporters and enhancing glycolytic rates, which results in an increase in lactate production (48,53,54). The glucose uptake process is regulated by glucose transporters. Adipocytes express glucose transporters from each class of the GLUT family (55). However, it is GLUT1 that is the main transporter for basal glucose uptake. In response to hypoxia in human adipocytes, GLUT1, GLUT3 and GLUT5 gene expression is increased, as well as 2-deoxy-D-glucose uptake (53,56). Additionally, HIF-1α is specifically involved in the increases in GLUT1 and glucose uptake (40,57). In order for cells to further adapt to oxygen restrictions, hypoxia also leads to increased glycogen accumulation in a HIF-1α dependent manner (58–60). In several cell types, this increase in glycogen storage by HIF-1α is mediated through glycogen synthase 1 (GYS1) (60). Additionally, in adipocytes and other cell types, hypoxia causes an up-regulation of genes that encode for enzymes in the glycolytic pathway (38,54,61). The glycolytic genes up-regulated by hypoxia include hexokinase 1 and 2 (HK1 and HK2), glucose-6 phosphate isomerase (G6PI), phosphofructokinase (PFK), aldolase A and C (ALDOA and ALDOC) , triosephosphate isomerase (TPI), phosphoglyceratekinase 1 (PGK1), enolase 1 and 2 (ENO1 and ENO 2) and lactate dehydrogenase (LDHA) (38,54,62). Not only are these key glycolytic enzymes up-regulated by hypoxia, they are dependent on HIF-1α (62).

The end product of the glycolytic pathway is pyruvate. In the presence of oxygen, pyruvate is converted into acetyl-CoA for entry into the TCA cycle for the production of NADH and $FADH_2$ for oxidative phosphorylation. Under anaerobic conditions, pyruvate is

converted into lactate and transported out of cells by monocarboxylate transporters (MCTs). During conditions of hypoxia, adipocytes have increased release of lactate, as well as increased gene expression of MCT1 and MCT4 (39,63). In both adipocytes and tumor cells, MCT4 is directly mediated by HIF-1α (64,65). In fact, the amount of glucose converted to lactate directly correlates with fat cell size, congruent with hypoxia increasing as adipose tissue expands (33). One effect of the export of lactate from the cell into the extracellular environment is an increase in the inflammatory response in macrophages, which provides one mechanism by which hypoxia and inflammation are associated (66).

Hypoxia and Inflammation. Obesity can be characterized by chronic, low-grade inflammation with increased circulating levels of inflammatory molecules (25). These inflammatory markers correlate with hypertrophic adipocytes, which are strongly associated with cardiometabolic risk (31). It is also this expansion of adipocytes that leads to hypoxia. In 2004, it was proposed that hypoxia initiates the inflammatory response in adipose tissue by directly dysregulating adipokine production (37). To investigate the link between hypoxia and inflammation, studies have examined the effect of low oxygen conditions and hypoxia mimetics on adipokine production. In human adipocytes, exposure to varying degrees of oxygen causes a dose-dependent increase in adipokine production. It has been shown that at 1% oxygen, there is a nine-fold increase in mRNA for leptin, 27-fold increase for VEGF, 22-fold increase in fasting-induced adipose factor (Angplt4/FIAF), 7.2-fold increase for IL-6 and a 6.6-fold increase for PAI-1. Additionally, anti-inflammatory adipokine, adiponectin is reduced by 3-fold under hypoxic conditions. The alterations in these mRNA levels are accompanied by an increase in the release of adipokines from the adipocytes. As oxygen levels decrease,

the secretion of leptin, VEGF, IL-6 and PAI significantly increase, while adiponectin secretion decreases (39) Moreover, the changes in adipokine expression and secretion by low oxygen conditions correspond to levels of HIF-1α and exposure to hypoxia mimetic, $CoCl_2$ (40). Exposure to $CoCl_2$ in murine adipocytes increases the level of protein of pro-angiogenic factors VEGF, leptin and matrix metalloproteinases, MMP-2 and MMP-9 (63). Many of these hypoxia-sensitive genes are dependent on the induction of HIF-1α (37).

It has been proposed that the increase of circulating adipokines in response to hypoxia is to stimulate angiogenesis and increase blood flow to the tissue (Fig.1.2). Angiogenesis is critical for the function of adipose tissue and underlies the process by which new blood vessels expand from existing vasculature. A major aspect of angiogenesis is the proliferation of endothelial cells, which is primarily regulated by VEGF-A, the master regulator of endothelial cell growth. In response to increased HIF-1α during hypoxic conditions in adipose tissue, increased VEGF transcription stimulates angiogenesis (67). In mice models, it has been shown that overexpression of VEGF improves the insulin resistance induced by a high fat diet (HFD). On the other hand, ablation of VEGF in adipose tissue increases inflammation and reduces glucose tolerance and insulin sensitivity (68). These studies confirm a link between hypoxia and inflammation, as well as a role for HIF-1α induced VEGF for adaptation to adipocyte hypertrophy and insulin resistance (Fig.1.2).

Inflammation and Cytokine Secretion. Chronic, low-grade inflammation found during obesity is associated with metabolic syndrome and an increased risk for type 2 diabetes and cardiovascular disease. Obesity-induced inflammation affects several organs within the body including adipose, pancreas, liver, muscle, heart and brain (6,25).

Since inflammation of adipose tissue is considered a hallmark of obesity and plays an important role in the development of insulin resistance, current hypotheses support the idea that adipose inflammation is the basis for metabolic syndrome (69). Both clinical and epidemiological studies have confirmed this connection. In 1995, Speigelman's group was the first to show that obese individuals have an increased expression and production of the cytokine TNF-α in adipose tissue and that it is positively correlated with insulin resistance (70). A reduction in body weight is associated with a decrease in TNF-α expression (71). Higher BMI, which is seen in those who have excess adipose tissue, causes an increase in C-reactive protein (CRP) concentrations (72). IL-6 induces CRP and both are associated with the development of type 2 diabetes (73). On the other hand, anti-inflammatory molecule adiponectin, which helps protect against metabolic syndrome, is decreased during obesity (74). These findings suggest that the balance between pro-inflammatory and anti-inflammatory adipokines is critical for normal metabolic function.

Adipose tissue in obese individuals has increased accumulation of immune cells, such as macrophages. Infiltration of macrophages within the adipose tissue is critical for the production of pro-inflammatory cytokines and is linked to insulin resistance (75). During obesity, there is an enrichment of macrophages and T lymphocytes, which cause a switch from M2 macrophage polarization to M1 macrophage polarization. Whereas M2 polarization maintains a healthy, anti-inflammatory state within the adipose tissue, M1 polarization leads to the secretion of pro-inflammatory cytokines such as TNFα, IL-6 and IL-β (76). These macrophage-secreted factors promote systemic inflammation and impair the ability of adipocytes to differentiate, which leads to insulin resistance (77).

Macrophage infiltration also increases the production of reactive oxygen species (ROS) and oxidative stress (69). ROS cause damage to cellular proteins, lipids and nucleic acids, as well contribute to the initiation of insulin resistance (78). Importantly, the pro-inflammatory cytokines produced from macrophage infiltration leads to mitochondrial dysfunction (69). For example, increased TNF-α causes significant changes in mitochondrial metabolism. Prolonged treatment of cultured adipocytes with TNF-α causes an increase in basal respiration and proton leak but a decrease in respiratory capacity. These changes occur in conjunction with an increase in extracellular acidification rate (ECAR), which measures the cell's dependence on anaerobic glycolysis (69). TNF-α and several other inflammatory markers, such as IL-6 and IL-1β, cause a dose-dependent increase in GLUT1 and GLUT3 mRNA, which is accompanied by an increase in glucose uptake (79). Additionally, TNF-α increases the production of ROS, which directly damage the mitochondria by decreasing ATP synthesis and dysregulating lipid homeostasis, leading to overall mitochondrial dysfunction (80). Collectively, these data demonstrate that increased inflammation and adipokine secretion in obesity directly impact mitochondrial metabolism and insulin resistance.

Oncostatin M. One family of cytokines, the IL-6 family, is a group of cytokines involved in various biological processes such as inflammation, proliferation, differentiation and immune response. The IL-6 family is also referred to as the glycoprotein 130 (gp130) family, because these cytokines rely on the gp130 receptor complex to mediate signal transduction (81). Recently, gp130 cytokines have been studied as potential therapeutic targets in obesity as research has shown that adipocytes and adipose tissue are sensitive to their secretion (82). One gp130 cytokine, Oncostatin

M (OSM), is unique because it also requires a special receptor, Oncostatin receptor β (OSMRβ) to mediate its effects (83,84). In fact, in the murine system, OSM only binds to the gp130/OSMRβ complex (85). OSM was originally identified and cloned in 1986 when it was found to be a cell growth regulator, having the ability to inhibit proliferation on the A375 human melanoma cell line (86).

The main producers of OSM include monocytes, macrophages, T cells, neutrophils and dendritic cells. The production and secretion of OSM leads to the activation of several signaling pathways. In fact, out of all IL-6 cytokines, OSM has the broadest signaling profile (85). This cytokine activates the JAK/STAT pathway, the mitogen-activated protein kinases (MAPK) ERK1/ERK2, the stress activated protein kinases p38 and c-Jun N-terminal pathway and protein kinase C delta (PKCδ) pathway (83,85). The activation of these signaling pathways by OSM leads to downstream effects in a variety of tissues and targets such as the liver, bone marrow, central/peripheral nervous system, muscle, heart, cancer and adipose tissue (84,85,87). Within the liver, OSM affects regeneration, differentiation and lipid metabolism (85). For example, OSM increases both the number of cell surface low density lipoprotein (LDL) receptors, as well as LDL uptake in a hepatoma cell line, which indicates a major role for OSM in cholesterol homeostasis within the liver (88). In regards to bone metabolism, several gp130 cytokines regulate both osteoblast and osteoclast activity (83). OSM increases bone colony numbers during the early proliferation phase of differentiation, but also causes an increase in apoptosis (89). Interestingly, the expression level of OSM increases throughout the progression of osteoblast differentiation, but OSMRβ expression decreases (90). Regulation of bone metabolism by OSM is sensitive to time and cell type (83). In skeletal muscle cells, OSM treatment induces growth arrest by

inhibiting proliferation, blocking cell cycle progression from G_1 to S phase and significantly reducing cyclin D1 protein levels (91). These data indicate the ability of OSM to cause growth arrest by regulating cell cycle.

OSM expression is also found in the central nervous system (CNS) originating in microglia, astrocytes, neurons and infiltrating leukocytes (83,92). OSM expression levels increase and correlate with several types of tumors, human immunodeficiency virus associated dementia (HAD), multiple sclerosis (MS) and epileptic seizures (92). Importantly, within the CNS, OSM has both pro- and anti-inflammatory actions, depending on the microenvironment. In fact, when OSM was first discovered for its' ability to inhibit cancer growth, researchers were examining OSM for anti-inflammatory properties for arthritis (83). However, it is the anti-proliferative effect of OSM in cancer cells that has been extensively studied over the last decade. OSM not only inhibits growth of human melanoma cell lines, it also regulates osteosarcoma proliferation and reduces proliferation of breast cancer in several cell lines (93,94). While OSM has a seemingly anti-proliferative role in cancer, some research suggests that it has a pro-tumorigenic role in some cell types (83). Collectively, OSM plays major roles in the regulation of a variety of important biological processes resulting in many physiological implications, which are dependent upon both species and cell type.

Oncostatin M in Adipose Tissue and Obesity. As mentioned previously, OSM is produced in several cells types such as T-cells and macrophages (85). Since T-cells and macrophages can be found in adipose tissue, this means OSM is produced within the adipose tissue. Although OSM is not specifically produced by preadipocytes or adipocytes, both cell types have OSM receptors, allowing them to be responsive to OSM (84). Research has shown that as humans become obese, OSM protein expression in

fat tissue increases. Additionally, OSM mRNA levels are increased in subcutaneous adipose tissue of obese individuals, compared to lean, and correlate with insulin levels, body weight and are inversely correlated with glucose disposal rate (87). Similarly, in epididymal adipose tissue from ob/ob mice, both the levels of OSM and OSMRβ are increased compared to lean littermates (87). In high fat-fed mice, OSM expression is significantly increased in adipose tissue T-cells and OSMRβ expression is increased following high fat feeding. OSM receptor knockout mice have increased insulin resistance and adipose tissue inflammation (95). These findings indicate that OSM is produced from adipose tissue, OSM levels are significantly increased during conditions of obesity and that OSM receptor levels are involved in the regulation of obesity.

Adipogenesis describes the process of fibroblasts and preadipocytes differentiating into mature adipocytes. Adipogenesis involves a cascade of transcription factors and changes in gene expression, which first lead to alterations in cell shape and ultimately lead to terminal differentiation (96,97). While there are a multitude of factors that play a regulatory role during early differentiation, a key underlying mechanism involves the quick induction of C/EBPβ and C/EBPδ, which are obligatory to drive expression of "master regulators" of adipogenesis, such as PPARγ and C/EBPα (96,98). Additionally, PPARγ and C/EBPα cross-regulate each other to drive differentiation (98). Terminal differentiation then allows preadipocytes to acquire the characteristics of fully mature adipocytes, including an increase in glucose transporters, insulin receptors and adipocyte proteins such as leptin and adipsin (96). It is well accepted that OSM inhibits adipocyte differentiation (81,84,99–101). In 3T3-L1 preadipocytes, OSM inhibits differentiation through the Ras/ERK and STAT5 signaling pathways. OSM exerts its effects by impacting the early phase of differentiation and may regulate C/EBPβ activity

(100). Additionally, OSM treatment reduces lipid content and the number of differentiated adipocytes, indicating a role for OSM in dedifferentiation of adipocytes (102). Inhibiting adipogenesis blocks fat cell expansion, which leads to insulin resistance (84).

OSM also regulates the expression and secretion of several adipokines. Exposure of 3T3-L1 adipocytes to OSM increases the expression of proinflammatory cytokines tissue inhibitor of metalloproteinases 1 (TIMP1), monocyte chemoattractant protein 1 (MCP1), insulin-like growth factor binding protein 3 (IGFBP3), osteopontin/secreted phosphoprotein1 (Spp1) and PAI-1. However, the induction of these adipokines was attenuated upon knockdown of OSMRβ, indicating a role for OSMRβ signaling in OSM-induced adipokine expression (95). OSM also has the ability to decrease adipokines, such as adiponectin. Adiponectin is an anti-inflammatory cytokine that is involved in regulating glucose levels and lipid metabolism. In fact, the concentration of adiponectin in plasma is decreased in obese and type 2 diabetic patients and reduced levels of adiponectin are related to insulin resistance (103). Therefore, the OSM-induced reduction of adiponectin is of particular significance.

As mentioned, angiogenesis is the process of forming new vasculature to continue supplying the expanding and proliferating adipocytes (67). Major regulators of angiogenesis include VEGF and to some extent, PAI-1 (67,104). As previously described, VEGF mediates the stimulation of endothelial cell proliferation (67,68,105). PAI-1, on the other hand, plays an important role in the fibrinolytic system and has also been shown to promote endothelial cell migration toward fibronectin, indicating a role for PAI-1 in angiogenesis (104,106). While both VEGF and PAI-1 are involved in the angiogenic response, VEGF seems to protect against high-fat diet induced obesity, improve insulin sensitivity and glucose tolerance, while PAI-1 strongly correlates with

BMI and is considered a risk factor for cardiovascular disease (106–109). Nonetheless, treatment of adipocytes with OSM increases both mRNA expression and protein production of VEGF and PAI-1. In both cases, OSM-induced VEGF and PAI-1 is dependent on the JAK/STAT pathway (106,110).

OSM and HIF-1α. Research indicates that cytokines and other growth factors can induce HIF-1α, the primary protein responsible for the various effects of hypoxia. For example, IFN-γ, IL-1β and TNF-α have a stimulatory effect on HIF-1 binding activity (111,112). In smooth muscle cells, angiotension II, thrombin and platelet-derived growth factor (PDGF) all strongly induce HIF-1α (113). Although hypoxia remains the incontestable inducer of HIF-1α, these data indicate that cytokines and growth factors have the ability to regulate HIF-1α even in normoxic conditions. Additionally, the activation of HIF-1α by these cytokines leads to many of the downstream effects caused by hypoxia (111–113). Of particular relevance, research indicates that in hepatic cells, OSM leads to the transcriptionally regulated induction of HIF-1α (114). As mentioned, OSM activates the JAK/STAT signaling pathway (83,85). It turns out that the induction of HIF-1α by OSM in liver cells is dependent on STAT3. Additionally, OSM stimulation leads to increased expression and secretion of both VEGF and PAI-1 and these increases are dependent on HIF-1α (114).

As indicated previously, hypoxia causes a switch from oxidative phosphorylation to anaerobic metabolism by increasing the expression of glycolytic genes and regulating glucose uptake and lactate production (48,53,54). In a human hepatocyte cell line, OSM was able to up-regulate HIF-1α under normoxic conditions and increase pyruvate dehydrogenase kinase 1 (PDK1) expression and protein levels (115). This data

correlates with other findings that HIF-1α specifically trans-activates the gene encoding PDK1 (51). PDK1 inhibits the conversion of pyruvate to acetyl CoA, which reduces mitochondrial respiration and subsequently increases the conversion from pyruvate to lactate (45). The result of an increase in PDK1 is a reduction in the oxidative TCA cycle flux (115). While research has not fully linked OSM to mitochondrial metabolic changes, some cytokines do lead to significant changes in mitochondrial bioenergetics. Treatment of 3T3-L1 adipocytes with TNF-α, IL-1β and IL-6 all result in changes of respiration profiles. These cytokines significantly change basal respiration, proton leak, ATP turnover, maximum capacity and basal extracellular acidification rate (ECAR). In particular, all cytokines lead to a reduction in maximum capacity and TNF-α significantly increases glucose uptake and basal ECAR due to an increase in lactate efflux (69). Mounting evidence indicates that hypoxia causes changes in adipocyte bioenergetics and other data reveal that OSM up-regulates HIF-1α and also impacts mitochondrial metabolism in some cell types. However, studies to link OSM and HIF-1α to adipocyte metabolism remain uninvestigated.

For terminal differentiation to occur, cells must go through mitotic clonal expansion (MCE), which is characterized by re-entry into cell cycle and several rounds of mitosis (96). Cell cycle is a tightly regulated process with four different phases Gap 1 (G1), synthesis (S), Gap 2 (G2) and mitosis (M) (116). The progression from G1 to S phase is a critical step as once cells enter S phase, they are committed to cell division (116,117). This transition depends on the E2F family of transcription factors, as well as increased cyclin-cyclin dependent kinase (CDK) activity (117). As mentioned, OSM inhibits adipocyte differentiation and in some cell types regulates cell cycle progression by inhibiting the transition from G1 to S phase (82,84,91). Research also indicates that

hypoxia inhibits adipocyte differentiation and this arrest is dependent on HIF-1α (118–121). Additionally, there is overwhelming evidence that in many cell types, hypoxia causes cell cycle arrest that is dependent on HIF-1α (116,122–125). For example, in mouse embryonic fibroblasts and splenic B lymphocytes, the deletion of HIF-1α inhibited the hypoxia-induced G1 arrest. In this case, the mechanism responsible for a G1 arrest is a HIF-1α dependent increase in p27 expression and hypophosphorylation of Rb (124). Similarly, in kidney epithelial cells, hypoxia caused cell cycle arrest at G1 phase by downregulating CDK2 activity, which correlated with p27 expression and hypophosphorylated RB (125). Collectively, it is clear that in many cell types HIF-1α regulates cell cycle progression, specifically at the G1/S phase transition (126). However, whether OSM-induced HIF-1α alters cell cycle progression during adipocyte differentiation is yet to be fully elucidated.

Review Summary. Obesity is a global health issue with major health consequences such metabolic disease, which encompasses type 2 diabetes and cardiovascular disease (3,6). Research indicates that obesity leads to hypertrophy, or expansion of adipocytes, within the adipose tissue and that this expansion leads to hypoxia, dysregulated adipokine secretion, inflammation and ultimately insulin resistance and type 2 diabetes (25,26,28). Evidence suggests that as adipocytes expand, the oxygen to adipose tissue doesn't increase proportionately, leading to a state of hypoxia. Ultimately, hypoxia causes functional changes such as a switch from oxidative metabolism to anaerobic metabolism and the regulation of cell cycle progression (33,34,37,38,126). Many of the effects of hypoxia are mediated through the transcription factor HIF-1α, which becomes stabilized in low oxygen conditions and regulates over 100 different genes (33,49). Interestingly, research has found that cytokine Oncostatin

M, which is produced from adipose tissue and increased during obesity, can induce HIF-1α and cause similar effects as hypoxia (84,87,114). While the relationship between OSM and HIF-1α has been established in some cell types, few studies have addressed their relationship and downstream effects in adipocytes. Therefore, we have proposed the following study objectives below to address our central hypothesis that OSM regulates adipocyte function and differentiation through mechanisms involving HIF-1α.

Study Objectives

Chapter II. It is well known that hypoxia causes functional changes within the adipose tissue during obesity. It is also established that there is a link between inflammation and hypoxia. Additionally, research demonstrates that OSM correlates with obesity and has the ability to up-regulate HIF-1α. However, the regulation and effects of OSM-induced HIF-1α have not been fully examined. Therefore, the objective of the study summarized in chapter II is to examine the role of HIF-1α in OSM-induced metabolic and angiogenic adaptations. We will determine 1) how HIF-1α is regulated by OSM, 2) the effect of OSM on glycolytic metabolism and angiogenic adipokine secretion, 3) a functional role for HIF-1α in OSM-mediated metabolic adaptations, as proposed by our working model (Fig.1.3).

Chapter III. Although evidence suggests that OSM affects adipocyte differentiation and cell cycle progression, a role for HIF-1α in this OSM-mediated process has not been investigated. Therefore, the objective of chapter III is to determine the role of OSM and OSM-induced HIF-1α in adipocyte cell growth, differentiation and cell cycle progression. We will 1) examine the regulation of preadipocyte growth and differentiation by OSM, 2) investigate the effect of OSM on cell cycle regulators, 3)

establish the role of HIF-1α in mediating OSM-induced adipose tissue remodeling as proposed by our working model (Fig.1.3).

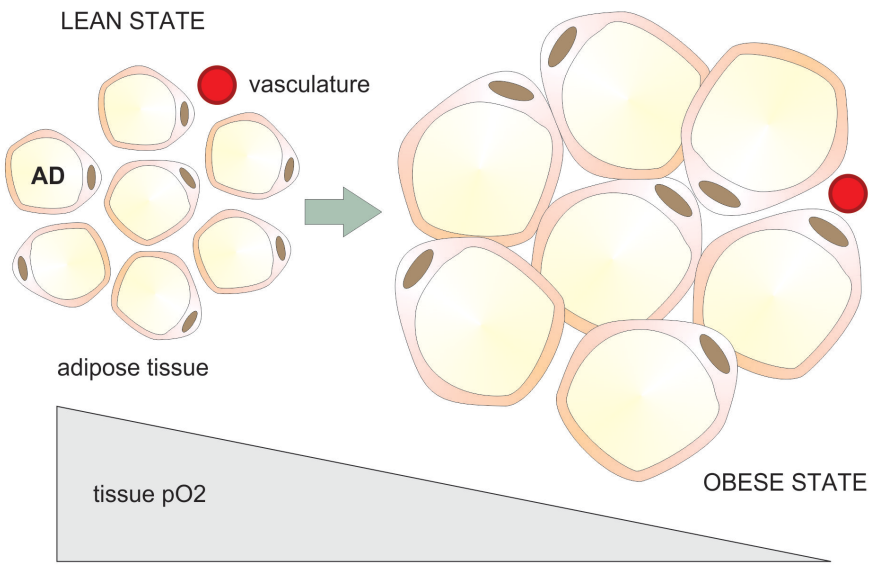

Figure 1.1. Oxygen Tension as Adipose Tissue Expands. As adipose tissue expands, the oxygen to the tissue does not increase proportionally, leading to a state of relative hypoxia.

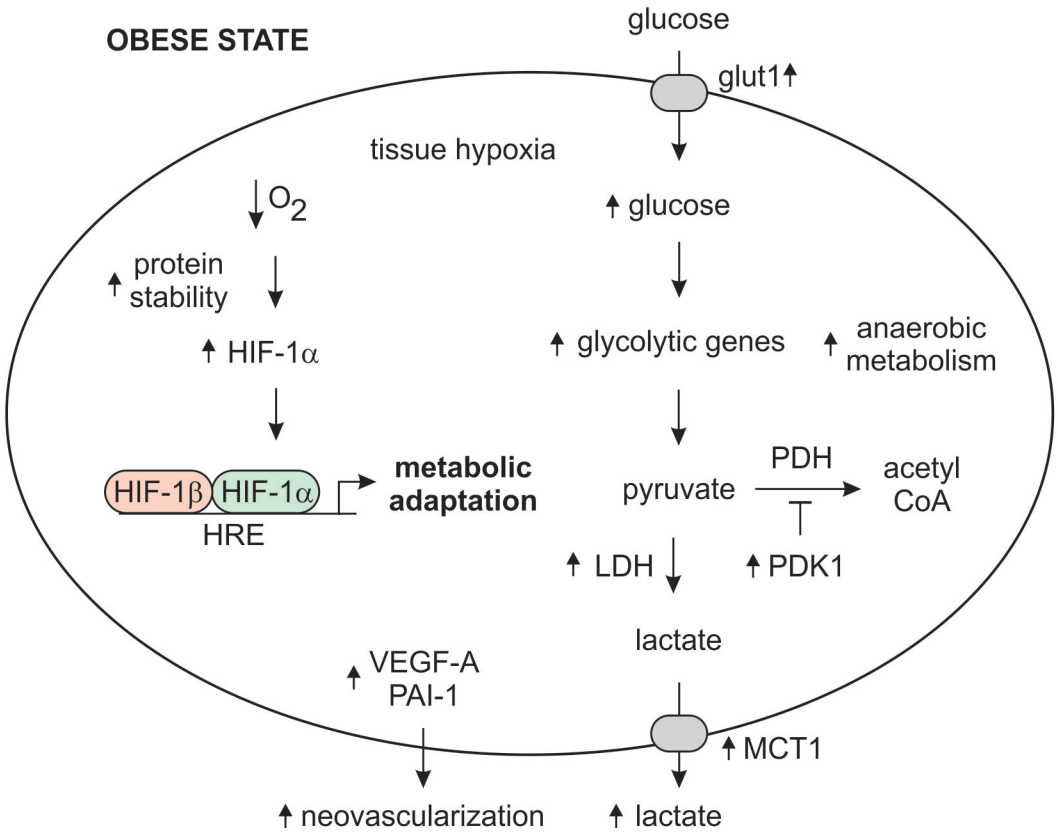

Figure 1.2. Stabilization of HIF-1α causes Metabolic Adaptations in Adipocytes. Stabilization of HIF-1α by hypoxia regulates the expression of genes involved in glycolysis, glucose uptake, lactate transport and neovascularization.

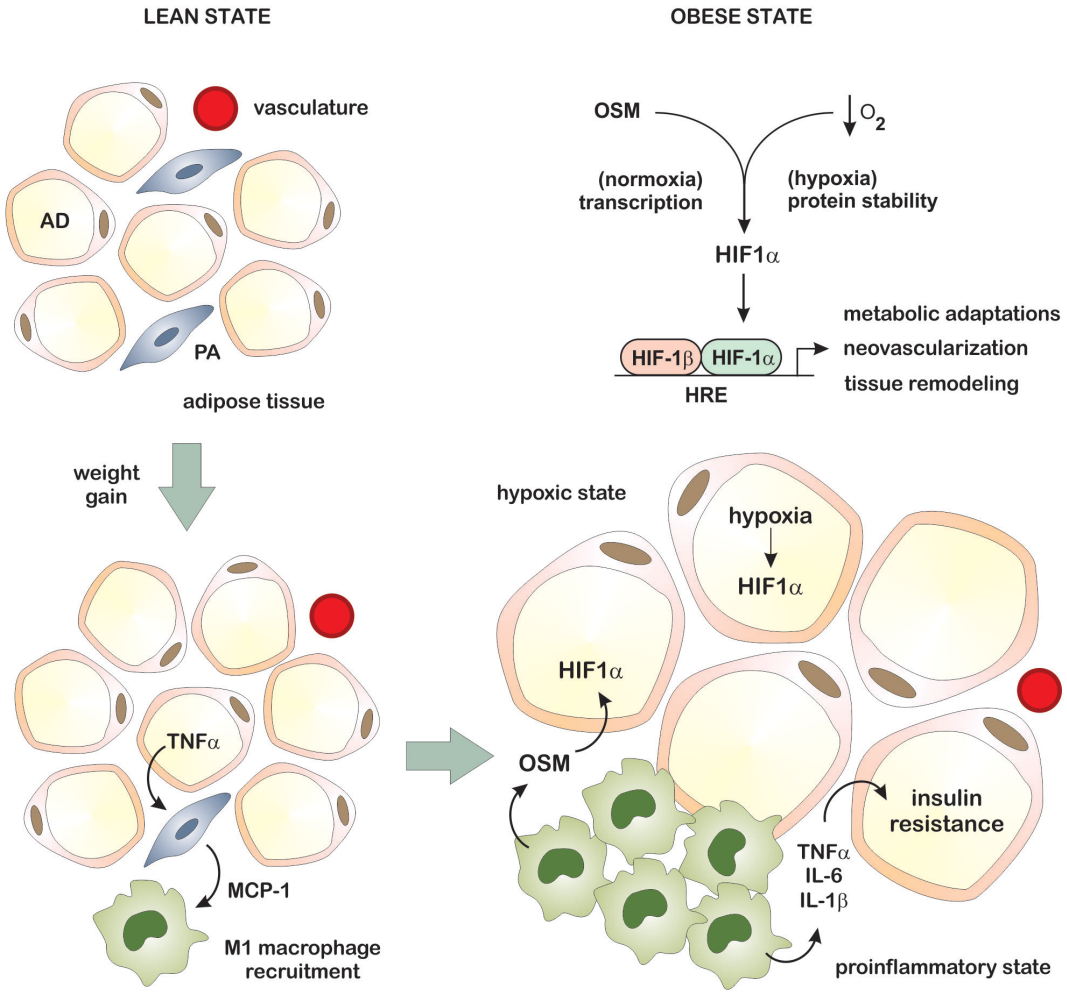

Figure 1.3. Working Model of the Regulation of HIF-1α by OSM and Synergistic Effects with Hypoxia. This model illustrates that the expansion of adipose tissue during obesity is accompanied with macrophage infiltration, inflammation and hypoxia. It predicts that the inflammatory cytokine OSM transcriptionally regulates HIF-1α and that the up-regulation of HIF-1α will occur in a synergistic manner with hypoxia and lead to downstream effects such as metabolic adaptations, neovascularization and tissue remodeling.

Reference List

1. Caballero B. The Global Epidemic of Obesity : An Overview. Epidemiol Rev. 2007;29(1):1–5.

2. Rush EC, Yan MR. Evolution not revolution: Nutrition and obesity. Nutrients. 2017;9(5):1–8.

3. Swinburn BA, Sacks G, Hall KD, McPherson K, Finegood DT, Moodie ML, et al. The global obesity pandemic: Shaped by global drivers and local environments. Lancet. 2011;378(9793):804–14.

4. Ogden CL, Carroll MD, Fryar CD, Flegal KM. Prevalence of Obesity Among Adults and Youth: United States, 2011-2014. NCHS Data Brief. 2015;(219):1–8.

5. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2014;384(9945):766–81.

6. Saltiel AR, Olefsky JM. Inflammatory mechanisms linking obesity and metabolic disease. J Clin Investig. 2017;127(1):4–7.

7. Jaspinder. K. A comprehensive review on metabolic syndrome. Cardiol Res Pract. 2014;2014:943162.

8. Centers for Disease Control and Prevention. Diabetes Report Card. Cent Dis Control Prev. 2012;(Cdc):16.

9. Sadikot SM, Das AK, Wilding J, Siyan A, Zargar AH, Saboo B, et al. Consensus recommendations on exploring effective solutions for the rising cost of diabetes. Diabetes Metab Syndr Clin Res Rev. 2017;11(2):141–7.

10. Kim DD, Basu A. Estimating the Medical Care Costs of Obesity in the United States: Systematic Review, Meta-Analysis, and Empirical Analysis. Value Heal. 2016;19(5):602–13.

11. Rutkowski JM, Stern JH, Scherer PE. The cell biology of fat expansion. Journal of Cell Biology. 2015.

12. Schoettl T, Fischer IP, Ussar S. Heterogeneity of adipose tissue in development and metabolic function. J Exp Biol. 2018;221(jeb162958):1–1

13. Ivanov S, Merlin J, Lee MKS, Murphy AJ, Guinamard RR. Biology and function of adipose tissue macrophages, dendritic cells and B cells. Atherosclerosis. 2018;271:102–10.

14. Ahima RS. Adipose Tissue as an Endocrine Organ. Obesity. 2006;14:242S–249S.

15. Mariman ECM, Wang P. Adipocyte extracellular matrix composition, dynamics and role in obesity. Cell Mol Life Sci. 2010;67(8):1277–92.

16. Samra JS, Clark ML, Humphreys SM, Macdonald I a, Frayn KN. Regulation of lipid metabolism in adipose tissue during early starvation. Am J Physiol. 1996;271(3 Pt 1):E541-6.

17. Wells JCK. The evolution of human adiposity and obesity: where did it all go wrong? Dis Model Mech. 2012;5(5):595–607.

18. Kennedy GC. The Role of Depot Fat in the Hypothalamic Control of Food Intake in the Rat Author. Proc R Soc London. 2016;140(901):578–92.

19. Coleman DL. Effects of parabiosis of obese with diabetes and normal mice. Diabetologia. 1973;9(4):294–8.

20. Zhang, Y; Proenca, R; Maffei, M; Barone, M; Leopold, L; Friedman J. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994;372:425–32.

21. Trayhurn P, Beattie JH. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. Proc Nutr Soc. 2001;60:329–39.

22. Matsuzawa Y. White adipose tissue and cardiovascular disease. Best Pract Res Clin Endocrinol Metab. 2005;19(4):637–47.

23. Sun K, Tordjman J, Clément K, Scherer PE. Fibrosis and adipose tissue dysfunction. Cell Metab. 2013;18(4):470–7.

24. Sun K, Kusminski CCM, Scherer PEP. Adipose tissue remodeling and obesity. J Clin Invest. 2011;121(6):2094–101.

25. Klöting N, Blüher M. Adipocyte dysfunction, inflammation and metabolic syndrome. Rev Endocr Metab Disord. 2014;15(4):277–87.

26. Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB. Adipose tissue remodeling: Its role in energy metabolism and metabolic disorders. Front Endocrinol (Lausanne). 2016;7(APR):1–16.

27. Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE. Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. Diabetologia. 2000;43(12):1498–506.

28. Hajer GR, Van Haeften TW, Visseren FLJ. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J. 2008;29(24):2959–71.

29. Goossens GH. The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. Physiol Behav. 2008;94(2):206–18.

30. Hauner H, Petruschke T, Russ M, Rohrig K, Eckel J. Effects of tumour necrosis factor alpha (TNF alpha) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture. Diabetologia. 1995;38(7):764–71.

31. Laforest S, Labrecque J, Michaud A, Cianflone K, Tchernof A. Adipocyte size as a determinant of metabolic disease and adipose tissue dysfunction. Crit Rev Clin Lab Sci. 2015;52(6):301–13.

32. Trayhurn P. Oxygen – the forgotten nutrient. J Nutr Sci. 2017;6:e47.

33. Trayhurn P. Hypoxia and Adipocyte Physiology: Implications for Adipose Tissue Dysfunction in Obesity. Annu Rev Nutr. 2014;34(1):207–36.

34. Trayhurn P. Endocrine and signalling role of adipose tissue: New perspectives on fat. Acta Physiol Scand. 2005;184(4):285–93.

35. Karpe F, Fielding B a, Ilic V, Macdonald I a, Summers LKM, Frayn KN. Response Is Related to Aspects of Insulin Sensitivity. Diabetes. 2002;2(18):2467–73.

36. Sun K, Halberg N, Khan M, Magalang UJ, Scherer PE. Selective Inhibition of Hypoxia-Inducible Factor 1 Ameliorates Adipose Tissue Dysfunction. Mol Cell Biol. 2013;33(5):904–17.

37. Wood IS, De Heredia FP, Wang B, Trayhurn P. Cellular hypoxia and adipose tissue dysfunction in obesity. Proc Nutr Soc. 2009;68(4):370–7.

38. Geiger K, Leiherer A, Muendlein A, Stark N, Geller-Rhomberg S, Saely CH, et al. Identification of hypoxia-induced genes in human SGBS adipocytes by microarray analysis. PLoS One. 2011;6(10).

39. Wood IS, Stezhka T, Trayhurn P. Modulation of adipokine production , glucose uptake and lactate release in human adipocytes by small changes in oxygen tension. Eur J Physiol. 2011;469–77.

40. Wang B, Wood IS, Trayhurn P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. Pflugers Arch Eur J Physiol. 2007;455(3):479–92.

41. Pasarica M, Rood J, Ravussin E, Schwarz JM, Smith SR, Redman LM. Reduced oxygenation in human obese adipose tissue is associated with impaired insulin suppression of lipolysis. J Clin Endocrinol Metab. 2010;95(8):4052–5.

42. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-Inducible Factors and the Response to Hypoxic Stress. Mol Cell. 2010;40(2):294–309.

43. Duan C. Hypoxia-inducible factor 3 biology: complexities and emerging themes. Am J Physiol Cell Physiol. 2016;310(4):C260–9.

44. Weidemann A, Johnson RS. Biology of HIF-1α. Cell Death Differ. 2008;15(4):621–7.

45. Semenza GL. Hypoxia-Inducible Factor 1: Regulator of Mitochondrial Metabolism and Mediator of Ischemic Preconditioning. Biochim Biophys Acta. 2012;1813(7):1263–8.

46. Dengler VL, Galbraith MD, Espinosa JM. Transcriptional regulation by hypoxia inducible factors. Crit Rev Biochem Mol Biol. 2014;49(1):1–15.

47. Jaakkola P, Mole DR, Tian Y, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of HIF-alpha to the von Hippel-Lindau Ubiquitylation Complex by Oxygen-Regulated Prolyl Hydroxylation. Science (80-). 2001;292(5516):468–72.

48. Eales KL, Hollinshead KER, Tennant DA. Hypoxia and metabolic adaptation of cancer cells. Oncogenesis. 2016;5(1):e190.

49. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 2003;3(10):721–32.

50. Chandel NS, Mcclintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, et al. Reactive Oxygen Species Generated at Mitochondrial Complex III Stabilize Hypoxia-inducible Factor-1 alpha during Hypoxia. J Biol Chem. 2000;275(33):25130–8.

51. Kim JW, Tchernyshyov I, Semenza GL, Dang C V. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. Cell Metab. 2006;3(3):177–85.

52. Fukuda R, Zhang H, Kim J whan, Shimoda L, Dang C V., Semenza GLL. HIF-1 Regulates Cytochrome Oxidase Subunits to Optimize Efficiency of Respiration in Hypoxic Cells. Cell. 2007;129(1):111–22.

53. Wood IS, Wang B, Lorente-Cebrián S, Trayhurn P. Hypoxia increases expression of selective facilitative glucose transporters (GLUT) and 2-deoxy-d-glucose uptake in human adipocytes. Biochem Biophys Res Commun. 2007;361(2):468–73.

54. Greijer AE, van der Groep P, Kemming D, Shvarts A, Semenza GL, Meijer GA, et al. Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor I (HIF-1). J Pathol. 2005;206(3):291–304.

55. Wood IS, Hunter L, Trayhurn P. Expression of Class III facilitative glucose transporter genes (GLUT-10 and GLUT-12) in mouse and human adipose tissues. Biochem Biophys Res Commun. 2003;308:43–9.

56. Wang B, Wood IS, Trayhurn P. PCR arrays identify metallothionein-3 as a highly hypoxia-inducible gene in human adipocytes. Biochem Biophys Res Commun. 2008;368(1):88–93.

57. Park HS, Kim J, Sun B, Song S, Suh W, Sung J. Hypoxia induces glucose uptake and metabolism of adipose-derived stem cells. Mol Med Rep. 2016;4706–14.

58. Mazure NM. Glycogen synthesis is induced in hypoxia by the hypoxia-inducible factor and promotes cancer cell survival. Front Oncol. 2012;2(February):1–9.

59. Ceperuelo-mallafré V, Ejarque M, Serena C, Duran X, Montori-grau M, Rodríguez MA, et al. Adipose tissue glycogen accumulation is associated with obesity-linked in fl ammation in humans. Mol Metab. 2016;5(1):5–18.

60. Pescador N, Villar D, Cifuentes D, Garcia-rocha M, Ortiz-barahona A, Cuevas Y, et al. Hypoxia Promotes Glycogen Accumulation through Hypoxia Inducible Factor (HIF)-Mediated Induction of Glycogen Synthase 1. PLoS One. 2010;5(3).

61. Mazzatti D, Lim F-L, O'Hara A, Wood IS, Trayhurn P. A microarray analysis of the hypoxia-induced modulation of gene expression in human adipocytes. Arch Physiol Biochem. 2012;118(3):112–20.

62. Iyer N V, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, et al. Cellular and developmental control of Oxygen homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev. 1998;149–62.

63. Lolmède K, Durand de Saint Front V, Galitzky J, Lafontan M, Bouloumié a. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. Int J Obes Relat Metab Disord. 2003;27(10):1187–95.

64. Heredia FP De, Wood IS. Hypoxia stimulates lactate release and modulates expression in human adipocytes. Eur J Physiol. 2010;509–18.

65. Luo F, Zou Z, Liu X, Ling M, Wang Q, Wang Q, et al. Enhanced glycolysis , regulated by HIF-1 α via MCT-4 , promotes inflammation in arsenite-induced carcinogenesis. Carcinogenesis. 2018;38(6):615–26.

66. Tan Z, Xie N, Banerjee S, Cui H, Fu M, Thannickal VJ, et al. The Monocarboxylate Transporter 4 Is Required for Glycolytic Reprogramming and Inflammatory Response in Macrophages. J Biol Chem. 2015;290(1):46–55.

67. Corvera S, Gealekman O. Adipose Tissue Angiogenesis: Impact on Obesity and Type-2 Diabetes. Biochim Biophys Acta. 2015;1842(3):463–72.

68. Sung HK, Doh KO, Son JE, Park JG, Bae Y, Choi S, et al. Adipose vascular endothelial growth factor regulates metabolic homeostasis through angiogenesis. Cell Metab. 2013;17(1):61–72.

69. Hahn WS, Kuzmicic J, Burrill JS, Donoghue MA, Foncea R, Jensen MD, et al. Proinflammatory cytokines differentially regulate adipocyte mitochondrial metabolism, oxidative stress, and dynamics. AJP Endocrinol Metab. 2014;306(9):E1033–45.

70. Hotamisligil GS, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest. 1995;95(5):2409–15.

71. Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB. The Expression of Tumor Necrosis Factor in Human Adipose Tissue. J Clin Endocrinol Metab. 1995;95(May):2111–9.

72. Visser M, Mcquillan GM, Wener MH, Harris TB. Elevated C-Reactive Protein Levels. J Am Med Assoc. 2018;282(22):2131–5.

73. Pradhan AD, Manson JE, Buring JE, Ridker PM. C-Reactive Protein, Interleukin 6, and Risk of Developing Type 2 Diabetes Mellitus. J Am Med Assoc. 2018;286(3):327–34.

74. Liang P. AdipoQ Is a Novel Adipose-specific Gene Dysregulated in Obesity*. J Biol Chem. 1996;271(18):10697–703.

75. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nat Rev Immunol. 2011;11(February):85–97.

76. Esser N, Legrand-Poels S, Piette J, Scheen AJ, Paquot N. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. Diabetes Res Clin Pract. 2014;105(2):141–50.

77. Taleb S, Keophiphath M, Miranville A, Clement K. Macrophage-Secreted Factors Impair Human Adipogenesis: Involvement of Proinflammatory State in Preadipocytes. Endocrinology. 2018;148(July):868–77.

78. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature. 2006;440(April):944–8.

79. Docanto, M, Ham S, Corbould A, Brown K. Obesity-Associated Inflammatory Cytokines and Prostaglandin E2 Stimulate Glucose Transporter mRNA Expression and Glucose Uptake in Primary Human Adipose Stromal Cells. J Interf Cytokine Res. 2015;35(8):600–5.

80. Chen XH, Zhao YP, Xue M, Ji CB, Gao CL, Zhu JG, et al. TNF-α induces mitochondrial dysfunction in 3T3-L1 adipocytes. Mol Cell Endocrinol. 2010;328(1–2):63–9.

81. White UA, Stewart WC, Stephens JM. Gp130 cytokines exert differential patterns of crosstalk in adipocytes both in vitro and in vivo. Obesity (Silver Spring). 2011;19(5):903–10.

82. White UA, Stephens JM. The gp130 Receptor Cytokine Family : Regulators of Adipocyte Development and Function. Curr Pharm Des. 2011;17(4):340–6.

83. Richards CD. The Enigmatic Cytokine Oncostatin M and Roles in Disease. ISRN Inflamm. 2013;512103.

84. Elks CM, Stephens JM. Oncostatin m modulation of lipid storage. Biology (Basel). 2015;4(1):151–60.

85. Hermanns HM. Oncostatin M and interleukin-31: Cytokines, receptors, signal transduction and physiology. Cytokine Growth Factor Rev. 2015;26(5):545–58.

86. Zarling JM, Shoyab M, Marquardt H, Hanson MB, Lioubin MN, Todaro GJ. Oncostatin M : A growth regulator produced by differentiated histiocytic lymphoma cells. Proc Natl Acad Sci. 1986;83(December):9739–43.

87. Sanchez-Infantes D, White UA, Elks CM, Morrison RF, Gimble JM, Considine R V., et al. Oncostatin M is produced in adipose tissue and is regulated in conditions of obesity and type 2 diabetes. J Clin Endocrinol Metab. 2014;99(2):217–25.

88. Grovef RI, Mazzucco CE, Radka SF, Shoyab M, Kiener PA. Oncostatin M Up-regulates Low Density Lipoprotein Receptors in HepG2 Cells by a Novel Mechanism*. J Biol Chem. 1991;266(Sep 25):18194–9.

89. Malaval LUC, Liu F, Vernallis ANNB, Aubin JE, Inserm U, Herriot E, et al. GP130/OSMR Is the Only LIF/IL-6 Family Receptor Complex to Promote Osteoblast Differentiation of Calvaria Progenitors. J Cell Physiol. 2005;(204):585–93.

90. Liu F, Aubin JE, Malaval L. Expression of Leukemia Inhibitory Factor (LIF)/ Interleukin-6 Family Cytokines and Receptors During In Vitro Osteogenesis : Differential Regulation by Dexamethasone and LIF. Bone. 2002;31(1):212–9.

91. Kim H, Jo C, Jang BG, Oh U, Jo SA. Oncostatin M induces growth arrest of skeletal muscle cells in G1 phase by regulating cyclin D1 protein level. Cell Signal. 2008;20(1):120–9.

92. Chen S, Benveniste EN. Oncostatin M: a pleiotropic cytokine in the central nervous system. Cytokine Growth Factor Rev. 2004;15:379–91.

93. Berreur M, Pradal G, Colombeix C, Heymann D. Downregulation of Osteoblast Markers and Induction of the Glial Fibrillary Acidic Protein by Oncostatin M in Osteosarcoma Cells Require PKC delta and STAT3. J Bone Miner Res. 2004;19:1850–61.

94. Douglas AM, Goss GA, Sutherland RL, Hilton DJ, Berndt MC, Nicola NA, et al. Expression and function of members of the cytokine receptor superfamily on breast cancer cells. Oncogene. 2010;14(1997):661–9.

95. Elks CM, Zha P, Grant RW, Hang H, Bailey JL, Burk DH, et al. Loss of Oncostatin M Signaling in Adipocytes Induces Insulin Resistance and Adipose Tissue Inflammation in Vivo*. J Biol Chem. 2016;291(33):17066–76.

96. Moreno-Navarrete JM, Fernández-real JM. Adipocyte Differentiation. Adipose Tissue Biology. 2012. 17-39 p.

97. Sarjeant K, Stephens JM. Adipogenesis. Cold Spring Harb Perspect Biol. 2012;4(9):1–19.

98. Brun R, Troy AE, Spiegelman BM, Darlington GJ, Wu Z, McKeon C, et al. Cross-Regulation of C/EBPα and PPARγ Controls the Transcriptional Pathway of Adipogenesis and Insulin Sensitivity. Mol Cell. 2004;3(2):151–8.

99. White UA, Stewart WC, Mynatt RL, Stephens JM. Neuropoietin attenuates adipogenesis and induces insulin resistance in adipocytes. J Biol Chem. 2008;283(33):22505–12.

100. Miyaoka Y, Tanaka M, Naiki T, Miyajima A. Oncostatin M inhibits adipogenesis through the RAS/ERK and STAT5 signaling pathways. J Biol Chem. 2006;281(49):37913–20.

101. Song HY, Jeon ES, Kim J Il, Jung JS, Kim JH. Oncostatin M promotes osteogenesis and suppresses adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells. J Cell Biochem. 2007;101(5):1238–51.

102. Song HY, Kim MR, Lee MJ, Jeon ES, Bae YC, Jung JS, et al. Oncostatin M decreases adiponectin expression and induces dedifferentiation of adipocytes by JAK3- and MEK-dependent pathways. Int J Biochem Cell Biol. 2007;39(2):439–49.

103. Hotta K, Funahashi T, Bodkin NL, Ortmeyer HK, Arita Y, Hansen BC, et al. Circulating concentrations of the adipocyte protein adponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in Rhesys Monkeys. Diabetes. 2001;50(May):1126–33.

104. Isogai C, Laug WE, Shimada H, Declerck PJ, Stins MF, Durden DL, et al. Plasminogen Activator Inhibitor-1 Promotes Angiogenesis by Stimulating Endothelial Cell Migration toward Fibronectin. Cancer Res. 2001;61:5587–94.

105. Mick GJ, Wang X, McCormick K. White Adipocyte Vascular Endothelial Growth Factor : Regulation by Insulin. Endocrinology. 2002;143(3):948–53.

106. Rega G, Kaun C, Weiss TW, Demyanets S, Zorn G, Kastl SP, et al. Inflammatory cytokines interleukin-6 and oncostatin M induce plasminogen activator inhibitor-1 in human adipose tissue. Circulation. 2005;111(15):1938–45.

107. Elias I, Franckhauser S, Ferré T, Vilà L, Tafuro S, Muñoz S, et al. Adipose tissue overexpression of vascular endothelial growth factor protects against diet-induced obesity and insulin resistance. Diabetes. 2012;61(7):1801–13.

108. Eriksson P, Reynisdottir S, Lönnqvist F, Stemme V, Hamsten a, Arner P. Adipose tissue secretion of plasminogen activator inhibitor-1 in non-obese and obese individuals. Diabetologia. 1998;41(1):65–71.

109. Juhan-Vague I, Alessi MC, Mavri A, Morange PE. Plasminogen activator inhibitor-1, inflammation, obesity, insulin resistance and vascular risk. J Thromb Haemost. 2003;1(7):1575–9.

110. Rega G, Kaun C, Demyanets S, Pfaffenberger S, Rychli K, Hohensinner PJ, et al. Vascular endothelial growth factor is induced by the inflammatory cytokines interleukin- 6 and oncostatin m in human adipose tissue in vitro and in murine adipose tissue in vivo. Arterioscler Thromb Vasc Biol. 2007;27(7):1587–95.

111. Haddad JJ, Harb HL. Cytokines and the regulation of hypoxia-inducible factor (HIF)-1 alpha. Int Immunopharmacol. 2005;5:461–83.

112. Scharte M, Han X, Bertges DJ, Fink MP, Delude RL, Han X, et al. Cytokines induce HIF-1 DNA binding and the expression of HIF-1-dependent genes in cultured rat enterocytes. Am J Physiol Gastrointest Liver Physiol. 2018;284:G373–84.

113. Richard DE, Berra E, Pouysségur J. Non-hypoxic pathway mediates the induction of hypoxia inducible factor 1 alpha (HIF-1α) in vascular smooth muscle. J Biol Chem. 2000;275(35):26765–71.

114. Vollmer S, Kappler V, Kaczor J, Flügel D, Rolvering C, Kato N, et al. Hypoxia-inducible factor 1α is up-regulated by oncostatin M and participates in oncostatin M signaling. Hepatology. 2009;50(1):253–60.

115. Battello N, Zimmer AD, Goebel C, Dong X, Behrmann I, Haan C, et al. The role of HIF-1 in oncostatin M-dependent metabolic reprogramming of hepatic cells. Cancer Metab. 2016;4(1):3.

116. Ortmann B, Druker J, Rocha S. Cell cycle progression in response to oxygen levels. Cell Mol Life Sci. 2014;71(18):3569–82.

117. Bertoli, C, Skotheim JM de BR. Control of cell cycle transition during G1 and S phase. Nat Rev Mol Cell Biol. 2013;14(8):518–28.

118. Lin Q, Lee YJ, Yun Z. Differentiation arrest by hypoxia. J Biol Chem. 2006;281(41):30678–83.

119. Trayhurn P. Hypoxia and Adipose Tissue Function and Dysfunction in Obesity. Physiol Rev. 2013;93:1–21.

120. Wang B, Wood IS, Trayhurn P. Hypoxia induces leptin gene expression and secretion in human preadipocytes: Differential effects of hypoxia on adipokine expression by preadipocytes. J Endocrinol. 2008;198(1):127–34.

121. Carrière A, Carmona MC, Fernandez Y, Rigoulet M, Wenger RH, Pénicaud L, et al. Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: A mechanism for hypoxia-dependent effect. J Biol Chem. 2004;279(39):40462–9.

122. Hubbi ME, Gilkes DM, Hu H, Kshitiz, Ahmed I, Semenza GL. Cyclin-dependent kinases regulate lysosomal degradation of hypoxia-inducible factor 1α to promote cell-cycle progression. Proc Natl Acad Sci USA. 2014;111(32):E3325-34.

123. Bae S, Jeong HJ, Cha HJ, Kim K, Choi YM, An IS, et al. The hypoxia-mimetic agent cobalt chloride induces cell cycle arrest and alters gene expression in U266 multiple myeloma cells. Int J Mol Med. 2012;30(5):1180–6.

124. Goda N, Ryan HE, Khadivi B, McNulty W, Rickert RC, Johnson RS. Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. Mol Cell Biol. 2003;23(1):359–69.

125. Krtolica a, Krucher N a, Ludlow JW. Hypoxia-induced pRB hypophosphorylation results from downregulation of CDK and upregulation of PP1 activities. Oncogene. 1998;17(18):2295–304.

126. Goda Nobuhito, Dozier Sara J, Johnson RS. HIF-1 in Cell Cycle Regulation, Apoptosis and Tumor Progression. Anal Chem. 2005;7(55):804–13.

CHAPTER II

REGULATION OF HIF-1α BY OSM IN ADIPOCYTES PROMOTES METABOLIC ADAPTATIONS

Abstract

Adipose tissue is a highly dynamic metabolic organ that plays a key role in energy homoeostasis. The expansion of adipose tissue results in a state of hypoxia, leading to the accumulation of hypoxia-inducible factor-1α (HIF-1α). Evidence suggests that hypoxia underlies the link between inflammation and adipocyte dysfunction. Recent studies demonstrate a key role for specific inflammatory cytokines in the regulation of HIF-1α and downstream effects. This study investigates the regulation of HIF-1α by the inflammatory cytokine, Oncostatin M (OSM), as well as a role for HIF-1α in OSM-mediated effects on glycolytic function and adipokine expression in 3T3-L1 adipocytes. Data presented here demonstrate that HIF-1α was induced by OSM at the level of gene expression and was dependent on ERK and AKT signaling pathways. Concurrent stimulation with hypoxia-mimetic, $CoCl_2$ and OSM caused a synergistic effect on HIF-1α, suggesting that during obesity, when both hypoxia and inflammation are present, there may be an amplifying effect on downstream targets of HIF-1α. Additionally, OSM increased glycolytic gene expression, glucose uptake and lactate production in a HIF-1α dependent manner. Finally, we show that OSM induction of angiogenic factors, vascular endothelial growth factor (VEGF) and plasminogen activator-1 (PAI-1), was dependent on HIF-1α. Collectively, these results suggest that HIF-1α has an essential role in the OSM-induced adaptations in adipocyte metabolism and vascularization during normoxia.

Introduction

Overweight and obesity are multifaceted diseases that are characterized by the expansion of adipose tissue and the increased risk of non-communicable diseases such as cardiovascular disease, stroke, diabetes and cancer (1,2). Although obesity is preventable, 36.5% of U.S. adults and 17% of U.S. youth were classified as obese in 2014 (3). Research indicates that the development of obesity-related diseases is, in part, regulated by white adipose tissue (WAT), which serves not only as an energy reservoir but also as a main secretory organ and site of inflammation (4). WAT is a highly metabolic organ that plays a critical role in energy homeostasis, obesity, inflammation and insulin resistance (5–7). As adipose tissue expands, the amount of oxygen to the tissue does not increase proportionally, leading to a state of tissue hypoxia or low oxygen tension, which results in various metabolic changes, as well as dysregulated adipokine production ((4,8).

Hypoxia activates the transcription factor, hypoxia-inducible factor-1α (HIF-1α), which has been proposed as a link between low oxygen tension and inflammation (8). HIF-1α is known as the “master regulator of oxygen homeostasis” (8–10). While HIF-1α is continually synthesized and degraded under normoxia, low oxygen conditions allow for stabilization by suppressing protein degradation (8,11). Once HIF-1α becomes stabilized, it translocates to the nucleus and heterodimerizes with HIF-1β for transcription of target genes (10,12). Due to the fact that many of the hypoxic effects are mediated through HIF-1α, researchers are able to study the effects of HIF-1α-dependent genes under normoxic conditions using chemical hypoxia mimetics, such as $CoCl_2$. HIF-1α is involved in the transcription of genes that encode for proteins involved in a variety of processes (8). Importantly, the induction of HIF-1α is a critical metabolic adaptation to

hypoxia due to its role in switching from oxidative to glycolytic metabolism (8,10,13). This switch allows for the maintenance of ATP production through increased anaerobic metabolism. For instance, during hypoxia, HIF-1α stimulates pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates pyruvate dehydrogenase (PDH), leading to the inhibition of the conversion from pyruvate to acetyl-CoA and results in reduced aerobic oxidation (14). HIF-1α is also specifically involved in increasing glucose transporter GLUT1, as well as glucose uptake in adipocytes (15,16). Additionally, in adipocytes and other cell types, hypoxia up-regulates genes that encode for enzymes in the glycolytic pathway (17–19). HIF-1α is also responsible for dysregulating adipokine production, which correlates with the inflammatory response (15,20,21).

It is well established that obesity is also associated with chronic, low-grade inflammation, which characterizes the term adipocyte dysfunction (22). In fact, adipose tissue inflammation may be the underlying basis for metabolic syndrome (23). The increase in pro-inflammatory cytokines leads to significant changes in mitochondrial metabolism, lipid homeostasis and insulin sensitivity (24,25). Of interest, research has shown that the gp130 cytokine, Oncostatin M (OSM), is increased as obesity increases. Specifically, OSM correlates with insulin levels, body weight and inversely correlates with glucose disposal rate (26). On the other hand, OSM receptor knockout mice have increased insulin resistance and adipose tissue inflammation (27). Evidence also suggests a role for OSM in the regulation and secretion of several adipokines (27–29).

More recently, there is growing interest in the link between hypoxia and inflammation. Research has shown that some cytokines, such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and OSM have the ability to increase HIF-1α, even under normoxic conditions (30,31). Evidence suggests that in

some cell types, OSM and other cytokines mediate processes typically regulated by hypoxia such as metabolic reprogramming, adipokine production and insulin signaling (23,32). For instance, in adipocytes, TNF-α and other cytokines significantly change respiration profiles and glucose uptake (23). In human hepatocytes, OSM up-regulates HIF-1α and increases PDK1 expression and protein levels, which results in a reduction in the oxidative TCA cycle flux (33). OSM stimulation in hepatic cells also leads to increased expression and secretion of VEGF and PAI-1 and HIF-1α dependent manner (32).

Although evidence indicates that hypoxia and OSM cause similar outcomes and the relationship between HIF-1α and OSM has been established in some cell types, relatively little attention has been given to its role in adipocytes. Since OSM has been reported to induce HIF-1α, we sought to elucidate the regulatory mechanisms of HIF-1α induction and subsequent effects on adipocyte function. We report here that OSM transiently induces functional HIF-1α at the level of transcription, rather than through protein stability as well known for hypoxia. Additionally, the induction of HIF-1α by OSM is dependent on ERK and AKT signaling pathways. Concurrent stimulation with hypoxia-mimetic, $CoCl_2$ and OSM causes a synergistic effect on HIF-1α, suggesting that during obesity, when both hypoxia and inflammation are present, there may be an amplifying effect on downstream targets of HIF-1α. Moreover, we observed that OSM increases glycolytic gene transcription, glucose uptake and lactate production and that this metabolic reprogramming is dependent on HIF-1α. Lastly, we revealed that OSM induces VEGF and PAI-1 transcription in a HIF-1α dependent manner. Collectively, our data indicate a role for HIF-1α in the OSM-changes in adipocyte metabolism and

adipokine secretion in a manner that supports a shift toward glycolytic metabolism and vascularization.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM), calf bovine serum (CS) and Trypsin-EDTA were purchased from Invitrogen. Murine Oncostatin M (OSM) was purchased for Sigma. The following antibodies were used for immunoblot analysis: HIF-1α, HIF-1β, GLUT1, phospho-ERK (Thr202/Tyr204), phospho-AKT (S-473), phospho-STAT3 (TYR705), total ERK and α-tubulin, were purchased from Cell Signaling. Pharmacological inhibitor of ERK (U0126) and pharmacological inhibitor of AKT (Wortmannin) were purchased from LC labs. Cycloheximide and Actinomycin D were purchased from Sigma. Dicer-substrate siRNA duplexes were purchased from Integrated DNA Technologies. Enhanced chemiluminescence (ECL) reagents were obtained from Perkin-Elmer Life Sciences. All TaqMan primer probes used in this study were purchased from Applied Biosystems.

Cell Culture. The murine 3T3-L1 cell line was purchased from Howard Green, Harvard Medical School. Cells were propagated in DMEM supplemented with 10% CS until reaching density-induced arrest, as previously described. Throughout the study, '0h' refers to density arrested cells immediately prior to stimulation as described in figure legend. Experiments described herein were conducted in density-arrested preadipocytes. All experiments were repeated 2- 3 times to validate results and ensure reliability.

Immunoblotting. Cell monolayers were washed with phosphate-buffer saline (PBS) and scraped into ice-cold lysis buffer containing 0.1 M Tris (pH 7.4), 150 mM NaCl, 10% sodium dodecyl sulfate (SDS), 1% Triton X, 0.5% Nonidet P-40 (NP40), 1

mM EDTA, 1 mM EGTA. Phosphatase inhibitor (2 μM sodium orthovanadate) and protease inhibitors (0.3 μM aprotinin, 21 μM leupetin, 1 μM pepstatin, 50 μM phenanthroline, 0.5 μM phenylmethylsulfonyl fluoride) were added to lysis buffer immediately prior to cell harvest. Cell lysates were sonicated and centrifuged (15,000*g,* 10 min, 4°C), and the supernatant transferred to a fresh tube. Protein content was determined by bicinchoninic acid (BCA) procedures according to manufacturer's (Pierce, Rockford, IL) instructions. Equal amounts of whole cell lysate protein were separated by SDS-PAGE electrophoresis. Cell lysates were mixed with loading buffer containing 0.25M Tris (pH6.8), 4% SDS, 10% glycerol, 0.01% bromophenol blue, and 10% dithiothreitol, then heated at 80°C for 5 min prior to electrophoresis. Proteins were resolved on SDS-polyacylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore corp., Billerica, MA). After transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4°C. Membranes were subsequently probed with horseradish peroxidase conjugated secondary antibodies for 2 hrs at room temperature. Membranes were immersed in ECL and data visualized by autoradiography using CL-XPosure film (Pierce).

Real-Time RT-PCR. Total RNA was extracted and genomic DNA contamination was removed using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), according to manufacturer protocol. Total RNA was quantified with a Nanodrop ND-1000 spectrophotometer. Total RNA was reverse-transcribed to cDNA in a 10 μl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems). The reverse transcription (RT) master mix containing RT buffer, deoxyribonucleotide triphosphate (dNTP) mix, RT random primers, RNase inhibitor (1.0 U/μl), and MultiScribe

RT was added to 1 µg RNA and RNase-free water. Reverse transcription reaction conditions followed the protocol (25°C for 10 min, 37°C for 120 min, 85°C for 5 sec, followed by 4°C in definitely/ RT complete) and utilized the Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for cDNA synthesis. PCR amplification was run utilizing the 7500 fast system (Applied Biosystems) that consisted of enzyme activation at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec combined with annealing /extension at 60°C for 30 sec. All data were analyzed with the ABI 7500 real time PCR system. All TaqMan primer probes used in this study were also purchased from ABI. Data were recorded and analyzed with ABI Sequence Detector Software and graphs visualized with Excel software. All data were presented as mean ± standard error of the mean (SEM) and representative of duplicate determinations. Data were normalized to 18S and measured as relative differences using the $2^{-\Delta\Delta CT}$ method as previously described (34,35). Statistical analyses were conducted using SPSS v25. Differences in glycolytic gene expression and angiogenic factors were determined via student's *t*-test where a p-value of <0.05 was considered significant. Knockdown data were analyzed using analysis of variance, with Tukey's post-hoc analysis used when the *p* value for the respective parameter was statistically significant ($p < 0.05$).

Co-immunoprecipitation. Co-immunoprecipitation (co-IP) was performed using the Thermo Fisher Scientific Pierce co-IP kit (26149) following the manufacturer's protocol. Briefly, total ERK and DUSP4 antibodies were first immobilized for 2 hrs using AminoLink Plus coupling resin. Cells were washed with PBS and incubated with IP Lysis/Wash buffer containing 25 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA and 5% glycerol on ice. Cell lysates were collected by brief centrifugation (13,000*g*, 10 mins, 4°C). The antibody-coupled resin was then washed and incubated with cell lysate

at 4°C overnight. After incubation, the resin was again washed and protein complex eluted using elution buffer. Protein complexes were then resuspended in loading buffer, resolved on SDS-polyacrylamide electrophoresis gel, and immunoblotted as discussed above.

Glucose Uptake. Glucose uptake was performing using the Glucose-Uptake Glo Assay (Promega) following the manufacturer's protocol. Briefly, cells propaged in 96-well culture dishes, grown until density-arrested and treated with OSM at several time points. Media was then removed from cells and washed with 100 μl of PBS. 50 μl of 1 mM 2DG was added, shaken briefly and incubated for 10 minutes at room temperature. After incubation, 25 μl of stop buffer was added and cells were shaken briefly. Following, 25 μl of neutralization buffer was added and cells were shaken briefly. Then, 100 μl of 2DG6P detection reagent was added, cells were shaken briefly and incubated for 1 hour at room temperature. Glucose uptake was then measured by luminescence (BioTek KC4). Statistical analyses were conducted using SPSS v25. Differences in glucose uptake were determined via student's *t*-test where a p-value of 0.05 was considered significant. Knockdown data were analyzed using analysis of variance, with Tukey's post-hoc analysis used when the *p* value for the respective parameter was statistically significant ($p < 0.05$).

Lactate Secretion. Extracellular lactate production was performed using the Lactate-Glo Assay (Promega) following the manufacturer's protocol. Briefly, cells were propagated in 96-well culture dishes, grown until density-arrested and treated with OSM at several time points. Media was then removed and diluted 40-fold in PBS. 50 μl of the sample was then transferred to a new 96-well assay plate and 50 μl of Lactate Detection Reagent was added to each sample. The plate was then shaken for 30 seconds and

incubated at room temperature. Lactate concentration was then recorded by luminescence (BioTek KC4). Statistical analyses were conducted using SPSS v25. Differences in lacate production were determined via student's *t*-test where a p-value of 0.05 was considered significant. Knockdown data were analyzed using analysis of variance, with Tukey's post-hoc analysis used when the *p* value for the respective parameter was statistically significant ($p < 0.05$).

RNA Interference. Dicer-substrate short interfering RNA (siRNA) for HIF-1α specific sequences as well as non-targeting sequences were transfected using Lipofectamine RNAiMAX transfection reagent according to manufacturer's (Invitrogen) protocol. Briefly, 3T3-L1 preadipocytes were propagated in 6-well culture dishes until reaching confluence. Cells were then stimulated with 9 μl Lipofecatime RNAiMAX reagent and either 10 μM HIF-1α specific siRNA or non-targeting siRNA for 48 hr. Cells were then stimulated with OSM and further analyses were carried out, as described above.

Results

Induction of HIF-1α by $CoCl_2$ and OSM is Dose and Time-Dependent.

Research has revealed that various cytokines have the ability to up-regulate HIF-1α (30,36). In fact, in a hepatic cell line, OSM significantly increases the induction of HIF-1α under normoxic conditions (32). Evidence demonstrates that the expansion of adipose tissue causes hypoxia, which leads to increased HIF-1α protein stability and that as humans become obese, OSM protein expression also increases (4,8,26). Although both hypoxia and OSM are regulated during conditions of obesity and a link between the two has been established in other cell types, little is known about the effect of OSM on HIF-1α in adipocytes. To first establish that the well-known hypoxia mimetic, $CoCl_2$ stabilizes

HIF-1α protein, we performed a time course and dose response. Preadipocytes (PAs) were either stimulated with 100μM $CoCl_2$ and harvested over time or stimulated with varying doses of $CoCl_2$ and harvested at 6 hr. Lysates were immunoblotted for HIF-1α and its well-known target, GLUT1. As illustrated in Fig.2.1B, HIF-1α was stabilized at 1 hr and continued to increase through 24 hr. Additionally, $CoCl_2$ had a dose-dependent effect on HIF-1α (Fig.2.1A). These increases were followed by a similar induction of GLUT1, which was both increased over time and in dose-dependent manner. To determine if OSM induces HIF-1α similar to $CoCl_2$, PAs were either stimulated with 1nM OSM and harvested over time or stimulated with varying doses and harvested at 6 hr. (Fig.2.1C&D). As shown in Fig. 2.1C, OSM increased HIF-1α and GLUT1 dose-dependently. Data also reveal (Fig.2.1D) that OSM increased HIF-1α to the same extent as $CoCl_2$, but in a transient manner. This was followed by an induction of the downstream HIF-1α target, GLUT1. These data indicate the estimated ED50 of OSM of 1nM leads to HIF-1α protein accumulation equivalent to that shown for the well-known hypoxia mimetic, $CoCl_2$.

OSM Induces HIF-1α at the Level of Transcription and not Protein Stability. It is well known that HIF-1α is continually synthesized and degraded under normoxia and that hypoxia or hypoxia mimetics, such as $CoCl_2$, enhance HIF-1α by blocking proteasomal degradation, leading to protein accumulation (8,10,37). In some cell types, OSM and other cytokines increase HIF-1α at the level of transcription (30–32,38). Mechanisms by which HIF-1α is regulated in adipocytes remain largely unknown. Thus, we explored mechanisms by which OSM increases HIF-1α protein in 3T3-L1 PAs. Density-arrested PAs were stimulated with OSM or $CoCl_2$ and examined for HIF-1α

mRNA and protein expression. Total RNA and whole cell lysates were collected over time and HIF-1α mRNA and protein expression was examined via RT-PCR and immunoblot analysis, respectively. As expected, $CoCl_2$ caused a sustained induction of HIF-1α protein (Fig.2.2A), but had no significant effect on mRNA expression of HIF-1α (Fig.2.2B). However, OSM significantly increased HIF-1α mRNA (Fig.2.2C) and protein expression in a transient manner (Fig.2.2A). These data suggest that OSM regulates HIF-1α at the level of transcription, while $CoCl_2$ regulates HIF-1α protein stability. Additionally, RT-PCR indicated that both $CoCl_2$ and OSM resulted in an increase in transcription of GLUT1 (Fig.2.2D&E), suggesting that induction HIF-1α by either agonist is functional. To confirm that OSM does not stabilize HIF-1α protein, HIF-1α was maximally induced in density-arrested PAs by 3 hrs OSM and $CoCl_2$ pretreatment and then stimulated with protein synthesis inhibitor cycloheximide (CHX) to prevent additional protein synthesis. Whole cell lysates were collected over time and immunoblotted for HIF-1α. As illustrated in Fig.2.3A, induction of HIF-1α by OSM was diminished by 1 hr, whereas induction of HIF-1α by $CoCl_2$ remained largely detectable at 1 hr. The faster rate of HIF-1α decay by OSM indicates that induction is not due to increased protein stability. To further confirm the regulation of HIF-1α by OSM, density-arrested PAs were stimulated concurrently with the transcription inhibitor, actinomycin D (AD) and OSM or $CoCl_2$. AD had no effect on HIF-1α protein in cells stimulated with $CoCl_2$, but eliminated HIF-1α levels in cells stimulated with OSM (Fig.2.3B). These data clearly demonstrate that OSM-mediated HIF-1α induction is due to transcription, while $CoCl_2$-mediated HIF-1α induction is due to protein stabilization.

OSM-Induced HIF-1α Interacts with HIF-1β to Form Functional Heterodimeric Complex. For HIF-1α to act as a functional transcription factor, it must be translocated to the nucleus and heterodimerized with HIF-1β (10,12,39). To establish the protein-protein interaction and confirm that the OSM-induced HIF-1α is functional, we stimulated density-arrested PAs with OSM, and harvested lysates at 6 hrs. Lysates were immunoprecipitated for HIF-1β and immunoblotted for HIF-1α and HIF-1β. As illustrated in Fig.2.4, under basal conditions where there was no OSM stimulation, HIF-1β did not co-immunoprecipitate with HIF-1α (lane 1). OSM stimulation for 6 hrs led to HIF-1α induction and HIF-1α-HIF-1β co-immunoprecipitation (lane 2). Non-specific binding of lysate proteins to agarose coupling resin was ruled out (lane 3). These data demonstrate that OSM-induced HIF-1α interacts with constitutively expressed HIF-1β to form the heterodimeric complex known to impart functional activity.

OSM Activates STAT, ERK and AKT Signaling Pathways. Previous research has established that OSM activates the janus kinase/signal transducer of activation (JAK/STAT), all three mitogen-activated protein kinases (MAPKs) and the (phosphoinositie-3-kinase-protein kinase/AKT (P13K/AKT) signaling pathways (40,41). The activation of these signaling pathways can have important implications. For example, some research suggests that OSM inhibits adipogenesis specifically through the RAS/ERK and STAT5 signaling pathways (42). Importantly, in liver cells, OSM-mediated HIF-1α up-regulation is dependent on STAT3 (32). To confirm that these well-known signaling pathways are activated in 3T3-L1 PAs, density-arrested cells were stimulated with OSM and whole cell lysates were collected over time. As shown in Fig.2.5, OSM caused a rapid increase in the phosphorylation of STAT3, ERK and AKT.

These data suggest that these specific signaling molecules may be involved in the downstream effects of OSM, such as changes in gene expression.

OSM-Induced HIF-1α is Dependent on ERK and AKT. Our data suggest that the phosphorylation of STAT3, ERK and AKT are all significantly increased with OSM. Previous studies have focused on the role of STAT3 in OSM-mediated HIF-1α regulation. Research has indicated that STAT3 is critical for the up-regulation of HIF-1α by OSM (32). However, there is a lack of research investigating a role for both ERK and AKT in OSM-mediated HIF-1α induction. Therefore, we sought to examine the importance of ERK and AKT in the up-regulation of HIF-1α. Density-arrested PAs were pretreated with MEK-inhibitor, U0126 or P13K-inhibitor Wortmannin for 1 hr to allow for complete inhibition of p-ERK and p-AKT, respectively. PAs were then stimulated with OSM and lysates were collected over time and immunoblotted for p-ERK, p-AKT, p-STAT3 and HIF-1α. As illustrated in Fig.2.6, the addition of U0126 reduced phosphorylation of ERK, as well as the levels of HIF-1α, indicating that p-ERK plays an important role in OSM-mediated HIF-1α up-regulation. Interestingly, the inhibition of p-ERK also affected the phosphorylation of AKT and STAT3, revealing that there is crosstalk between signaling pathways. In Fig.2.7, data show that pretreatment with Wortmannin completely inhibited the phosphorylation of AKT, which greatly reduced HIF-1α induction. Inhibition of AKT also altered the phosphorylation of ERK and STAT3, reiterating our finding that there is crosstalk between signaling pathways. To further emphasize the importance of the ERK and AKT pathways in OSM-mediated induction of HIF-1α, PAs were pretreated with the combination of U0126 and Wortmannin for 1 hr before stimulation with OSM and cell lysates were collected over time and immunoblotted for p-ERK, p-AKT, p-STAT3 and HIF-1α. As illustrated in Fig.2.8,

phosphorylation of both ERK and AKT were significantly reduced, while there was little change in p-STAT3. Although p-STAT3 remained unchanged, the inhibition of ERK and AKT, nearly abolished OSM-induced HIF-1α. These data confirm ERK and AKT as critical signaling molecules for the up-regulation of HIF-1α by OSM, independent of STAT3.

$CoCl_2$ and OSM have a Synergistic Effect on HIF-1α. Data presented above demonstrate that both $CoCl_2$ and OSM induce HIF-1α but do so by different mechanisms. Research indicates that HIF-1α regulates genes involved in a variety of processes such as angiogenesis, inflammation, proliferation, differentiation and glucose metabolism (8,13,43,44). Therefore, we hypothesized that if $CoCl_2$ and OSM induce HIF-1α by different mechanisms, the stimulation of PAs with both $CoCl_2$ and OSM would cause a synergistic effect on HIF-1α. An even greater increase in HIF-1α protein would likely lead to an increase in the downstream effects of HIF-1α seen during conditions of obesity when both hypoxia and inflammation are present. To test this hypothesis, density-arrested PAs were stimulated with $CoCl_2$ and OSM in conjunction and whole cell lysates were collected over time and immunoblotted for HIF-1α, as well as GLUT1. As illustrated in Fig.2.9, immunoblotting reveals that stimulation with $CoCl_2$ and OSM caused a synergistic effect on HIF-1α and GLUT1. These data suggest that during obesity, when both hypoxia and inflammation are present, the synergistic effect of these conditions on HIF-1α could lead to an amplifying effect on downstream targets of HIF-1α.

OSM Increases Glycolytic Gene Transcription, Glucose Uptake and Lactate Secretion. OSM is a pleiotropic cytokine that has been implicated in inflammation,

differentiation, insulin signaling, lipid metabolism and more recently has been shown to be involved in the metabolic reprogramming in some cell types (26,33,45). This is in congruence with research suggesting that some cytokines, such as TNF-α and IL-6, have the ability to regulate mitochondrial bioenergetics. (23) Additionally, our preliminary data indicate that OSM increases GLUT1 (Fig.2.1D), which suggests that OSM may shift from oxidative to glycolytic metabolism. Therefore we investigated the effect of OSM on the enzymes within the glycolytic pathway, as well as the effect on glucose uptake and lactate production. PAs were stimulated with OSM and total RNA was collected over time and analyzed for various glycolytic genes. As illustrated in Fig.2.10, OSM significantly up-regulated all of the genes encoding for enzymes in the glycolytic pathway. While we were unable to determine the effect of OSM on G6P isomerase, OSM increased hexokinase-2 (HK2), phosphofructokinase-1 (PFKL), aldolase A (ALDOA), trieosphosphate isomerase (TPI1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK1), phosphoglycerate mutase (PGAM1), enolase A (ENOA) and pyruvate kinase-M (PKM). Additionally, OSM increased gene expression of pyruvate dehydrogenase kinase 1 (PDK1), which inhibits pyruvate dehydrogenase (PDH) from converting pyruvate to acetyl CoA for entrance into the TCA cycle. This is consistent with the finding that OSM increases lactate dehydrogenase-A (LDHA), as well as glucose transporter GLUT1 and lactate transporters monocarboxylate 1 and 4 (MCT1, MCT4) (Fig.2.11). These data indicate that OSM transcriptionally up-regulates enzymes in the glycolytic pathway, as well as enzymes involved in anaerobic metabolism, such as glucose and lactate transporters (Fig.2.12). We further sought to determine whether the effect of OSM on glycolysis led to changes in glucose uptake and lactate secretion. PAs were stimulated with OSM and

glucose uptake was analyzed over time by luminescence. Data further revealed that OSM significantly increased glucose uptake (Fig.2.13). Moreover, stimulation of PAs with OSM for 24 hr led to an increase in lactate secretion as measured by luminescence (Fig.2.14). Collectively, these data suggest that OSM affects adipocyte bioenergetics by increasing anaerobic metabolism as would be expected for hypoxia, but not under normoxic conditions.

OSM-Induced HIF-1α is Involved in the Metabolic Reprogramming of Adipocytes. A well-known adaptation to hypoxia is the switch from oxidative to glycolytic metabolism through HIF-1α (8,10,46). When too little oxygen is present, aerobic metabolism is decreased, so in order to maintain ATP production, HIF-α increases anaerobic oxidation (46). Our data suggest that OSM causes a switch from oxidative to glycolytic metabolism. In hepatocytes, OSM is able to alter metabolic parameters in a HIF-1α-dependent manner (33). However, research has not fully linked OSM and HIF-1α to metabolic changes in adipocytes. Therefore, we examined the role for HIF-1α in OSM-mediated effects on metabolic reprogramming. PAs were transfected with siRNA targeted to HIF-1α or non-targeting control sequences for 48 hr prior to stimulation with OSM. Total RNA and whole cell lysates were harvested over time and analyzed for mRNA abundance of HIF-1α and the previously described glycolytic genes. As illustrated in Fig.2.16, each gene was analyzed at a time that fit the peak of induction as observed from previous data. Data indicated that HIF-1α gene expression (Fig.2.15A) and protein (Fig.2.15B) was markedly induced by OSM in cells transfected with control siRNA. In contrast, induction of HIF-1α mRNA and protein was significantly suppressed in cells treated with HIF-1α targeting siRNA (Fig.2.15A). As illustrated in Fig.2.15C,

knockdown of HIF-1α significantly reduced the OSM-mediated up-regulation of the genes encoding enzymes in the glycolytic pathway including HK2, PFKL, ALDOA, TPI1, GAPDH, PGK1, PGAM1, ENOA, as well as PDK1, compared to cells transfected with non-targeting siRNA and stimulated with OSM. The only gene in the glycolytic pathway not affected by siRNA targeted to HIF-1α was PKM. Additionally, HIF-1α knockdown significantly reduced the OSM-mediated increases in GLUT1, LDHA and MCT4. In contrast to these findings, HIF-1α knockdown significantly increased OSM-mediated up-regulation of MCT1 compared to non-targeting siRNA. To further support a role for HIF-1α in the OSM-induced shift in metabolism toward glycolytic flux, PAs were transfected with siRNA targeted to HIF-1α or non-targeting control sequences for 36 hr prior to stimulation with OSM. Glucose uptake was measured by luminescence at 12 hr post-OSM treatment. As shown in Fig.2.16, glucose uptake was significantly increased in cells transfected with control siRNA, while knockdown of HIF-1α prevented the OSM-mediated increase in glucose uptake. Similarly, PAs were transfected with both siRNA for HIF-1α and non-targeting control for 24 hr before treatment with OSM and lactate secretion was measured by luminescence at 24 hr post-OSM stimulation. Control siRNA cells treated with OSM had a significant increase in lactate secretion, while cells transfected with siRNA for HIF-1α and treated with OSM did not significantly increase lactate secretion compared to cells with HIF-1α siRNA only (Fig.2.17). These data suggest that the OSM-mediated increase in glycolytic function, including an increase in glycolytic gene transcription, glucose uptake and lactate secretion, is dependent on HIF-1α.

OSM Stimulation Increases VEGF and PAI-1. It is well accepted that increasing obesity leads to dysregulated adipokine production (20). These adipokines

are involved in a variety of physiological processes such as glucose homeostasis, insulin sensitivity, lipid metabolism and angiogenesis (47,48). The capacity of adipose tissue to continually expand requires a proportional increase in vascularization to meet its increasing energy demands (49). The major regulator of this vascularization is VEGF. In particular, it is well accepted that VEGF-A increases vascularization in white adipose tissue (50,51). On the other hand, VEGF-B acts as a vascular survival factor and VEGF-C and VEGF-D are mainly involved in lymphangiogenesis (52). Research has shown that in human adipocytes, OSM increases mRNA and protein production of VEGF. To confirm that OSM increases VEGF in our 3T3-L1 model, density-arrested PAs were stimulated with OSM and total lysates were collected over time and analyzed for relative VEGF-A mRNA abundance. Data revealed the OSM significantly induces VEGF-A in a transient manner but had no effect on VEGF-B or VEGF-C (Fig.2.18). Research also indicates that OSM induces PAI-1, an adipokine involved in both angiogenesis and insulin resistance (28,53,54). To elucidate the impact of OSM on PAI-1, PAs were stimulated with OSM and total RNA was collected over time and analyzed for relative PAI-1 abundance. Similar to the effect on VEGF-A, treatment with OSM caused a transient induction of PAI-1 (Fig.2.18).

Role for HIF-1α in OSM-Induced Expression of VEGF and PAI-1. Data presented above demonstrate that OSM leads to dysregulated adipokine production. Research also shows that hypoxia regulates the expression of adipokines, such as VEGF, PAI-, IL-6, MMP-2 and MMP-9, and that many of these hypoxia sensitive genes are dependent on the induction of HIF-1α (15,20,21,55). To investigate a role for HIF-1α in OSM-mediated alterations in adipokine expression, PAs were transfected with siRNA targeted to HIF-1α or non-targeting control sequences for 48 hr before stimulation with

OSM. Total RNA was collected over time and analyzed for mRNA abundance of VEGF-A and PAI-1 at a time that fit the peak of induction as observed from previous data. Data indicated that HIF-1α gene expression was markedly induced by OSM in cells transfected with control siRNA. However, as illustrated in Fig.2.19, specific knockdown of HIF-1α significantly reduced the OSM-mediated up-regulation of VEGF-A and PAI-1 compared to control. These data demonstrating that OSM stimulated VEGF-A and PAI-1 expression is dependent on HIF-1α support the premise that hypoxia is directly linked to inflammation and an angiogenic response.

Discussion

In this investigation, we demonstrated a role for HIF-1α in OSM-mediated regulation of gene expression in 3T3-L1 adipocytes. First, we report that induction of HIF-1α by OSM and hypoxia mimetic $CoCl_2$ is dose and time dependent. Importantly, HIF-1α was induced to the same extent by OSM as $CoCl_2$. Second, we show that the induction of HIF-1α by OSM is regulated at the level of transcription through STAT, ERK and AKT signaling pathways. Of these signaling pathways, OSM-induced HIF-1α was dependent on ERK and AKT. Third, we confirm that HIF-1α interacts with HIF-1β to form a functional heterodimeric complex and act as a transcription factor. Fourth, we found that the addition of both $CoCl_2$ and OSM has a synergistic effect on HIF-1α. Fifth, we present evidence that OSM increases glycolytic gene transcription, glucose uptake and lactate secretion and that HIF-1α is involved in this metabolic reprogramming of adipocytes. Finally, we show that OSM increases the expression of VEGF and PAI-1 and that HIF-1α plays a role in the induction of these angiogenic adipokines. Collectively, data presented in this report demonstrate that OSM transcriptionally

regulates HIF-1α and OSM regulates glycolytic gene expression, glucose uptake, lactate production and angiogenic adipokine expression in a HIF-1α dependent manner in 3T3-L1 adipocytes.

It is well established that both hypoxia and inflammation are present in obese adipose tissue and that these conditions lead to dysregulated glucose and lipid metabolism, insulin resistance and overall adipocyte dysfunction (56–60). It's been proposed that hypoxia initiates the inflammatory response in adipose tissue (20). Hypoxic conditions contribute to many functional changes through the activation of transcription factor, HIF-1α (8). Investigations in the past have extensively focused on the effects of HIF-1α on adipokine secretion (15,21). Accumulating evidence now suggests that adipokines have the ability to regulate HIF-1α, even under normoxic conditions (30,31,38). Recent studies have shown that as obesity increases, the inflammatory cytokine, OSM increases and correlates with obesity and insulin sensitivity (26). Data in this investigation present evidence that OSM increases the abundance of HIF-1α mRNA and protein, supporting the premise that HIF-1α can be regulated by adipokines under normoxic conditions. In comparison to the up-regulation of HIF-1α by $CoCl_2$, which was sustained, induction of HIF-1α by OSM was transient. However, we found that HIF-1α protein abundance induced by OSM was increased to the same extent of $CoCl_2$, which demonstrates the significance of the impact of OSM on this transcription factor. While the regulation of HIF-1α by hypoxia is well established, mechanisms by which cytokines induce HIF-1α remain poorly understood. Data presented above demonstrate that OSM increases HIF-1α mRNA, but does not effect protein stability. Blockade of transcription with Actinomycin D inhibited OSM induced HIF-1α, but had no

effect on HIF-1α protein induced by $CoCl_2$. Consistent with our observation, Vollmer and colleague also reported that OSM induces HIF-1α in a transcription-dependent manner in hepatocytes (32). In some cell types, it has been shown that the HIF-1α induction by cytokines is a functional transcription factor (30–32,38). Our data confirm that OSM-induced HIF-1α interacts with HIF-1β to form a functional heterodimeric complex. Taken together, our data demonstrate that the induction of HIF-1α by OSM is regulated at the level of transcription and activates a fully functional transcription factor, which suggests that the effects of OSM on adipocyte function may be related, in part, to the induction of HIF-1α.

Evidence from previous studies indicates that OSM exerts its pleiotropic effects through a variety of signaling pathways. For example, OSM has been reported to activate the JAK/STAT, (MAPK) ERK1/ERK2, p38, c-Jun N-terminal, and protein kinase C delta (PKCδ) signaling pathways (32,40,41). Our findings demonstrate that OSM quickly increases STAT, ERK and AKT signaling pathways and may be involved in the induction of HIF-1α by OSM. Previous research has concentrated on the activation of the JAK/STAT pathway, including both STAT3 and STAT5, in response to OSM (27,32). Moreover, Vollmer and colleague have found that the induction of HIF-1α by OSM is dependent on STAT3 phosphorylation (32). In contrast, we found that the suppression of both ERK and AKT with U0126 and Wortmannin, respectively, completely inhibited the induction of HIF-1α by OSM, independent of STAT3. Collectively, these data suggest that ERK and AKT are critical signaling molecules for the OSM-mediated induction of HIF-1α.

Adipocyte dysfunction is characterized by a conglomeration of factors that are implicated during obesity such as reduced insulin sensitivity, increased hypoxia, increased adipokines, increased apoptosis, increased autophagy and increased reactive oxygen species (ROS) (56). Like hypoxia, hypoxia-mimetic, $CoCl_2$ stabilizes the HIF-1α protein, whereas our data show that OSM increases HIF-1α at the level of transcription. Our data demonstrate that the combination of both $CoCl_2$ and OSM has a synergistic effect on HIF-1α, causing a greater increase in the abundance of this transcription factor with the combination of treatments than either treatment alone. While no other study has addressed the amplifying effect of both hypoxia and OSM on HIF-1α in adipocytes, these data suggest that during adipose tissue expansion, the increase and downstream effects of HIF-1α on adipocyte dysfunction may also be attributed to inflammation, not just hypoxia.

It is well established that a critical metabolic adaptation to hypoxia is the HIF-1α-dependent switch from oxidative to glycolytic metabolism (8,10,46). HIF-1α increases glycolytic metabolism through a variety of mechanisms including increased PDK1 activity, induction of GLUT1, GLUT3, GLUT5, increased glucose uptake, regulation of glycolytic gene expression, as well as increased expression of lactate secretion and MCT1 and MCT4 expression (14,16–18,21,55,61,62). More recently, studies have found that some cytokines, such as TNF-α, IL-6 and IL-1β increase glucose transporters, glucose uptake, cause changes in basal respiration and increase lactate efflux (23,63). Data presented in this investigation reveal for the first time that OSM significantly up-regulated all genes encoding for enzymes in the glycolytic pathway, as well as PDK1, LDHA, GLUT1, MCT1 and MCT4. Survival of cells during hypoxia requires the downstream targets of HIF-1α to be up-regulated to cope with hypoxic stress. Greijer

and colleague found that HIF-1α significantly regulated all glycolytic enzymes (18). Our data provides evidence that HIF-1α is involved, in part, in the OSM-mediated induction of glycolytic enzymes. Not all enzymes up-regulated by OSM were dependent on HIF-1α, which may be explained by the fact that OSM is a pleiotropic cytokine. Furthermore, we found that siRNA targeted to HIF-1α without OSM stimulation significantly reduced the level of gene expression of glycolytic enzymes. This raises the possibility that several of these glycolytic enzymes depend on a basal level of HIF-1α to stay up-regulated. Previous research suggests that hypoxia initiates the hypoxic response. However, our data suggest that inflammation initiates the hypoxic response, via HIF-1α. Furthermore, we found that OSM led to an increase in glucose uptake, as well as lactate secretion, both of which were dependent on the induction of HIF-1α. Therefore, these data demonstrate that the OSM-mediated switch to glycolytic function is dependent on HIF-1α. This finding suggests that adipocytes with increased levels of OSM require the induction HIF-1α for survival during inflammatory stress.

It is widely accepted that hypoxia causes an increase in adipokine production and that changes in the expression of these adipokines correspond to the levels of HIF-1α (15,20). For instance, exposure to low O_2 conditions in adipocytes increases VEGF, IL-6 and PAI-1. (21) It has been hypothesized that the effect of hypoxia on adipokine production is to stimulate angiogenesis for increased blood flow to the adipose tissue. VEGF and to some extent, PAI-1 are major regulators of angiogenesis. Interestingly, treatment of adipocytes with OSM increases both VEGF and PAI-1 (28,29). Consistent with these findings, our data indicate that OSM increases VEGF and PAI-1 and are dependent on HIF-1α for induction. These data suggest that HIF-1α is the mechanism

by which OSM up-regulates VEGF and PAI-1 and that these adipokines may be induced to increase angiogenesis for metabolic adaptation to hypoxic and inflammatory stress. Based on these findings, we developed the following working model (Fig.2.20), where hypoxia and inflammation during obesity lead to the accumulation of HIF-α to regulate metabolic adapation.

In summary, this study is the first investigation showing that the transcriptional regulation of HIF-1α by OSM leads to changes in mitochondrial bioenergetics and adipokine expression in adipocytes. Previous research has shown that both hypoxia and increased OSM expression is present under conditions of obesity. Data presented here show that OSM leads to a transient, yet strong induction of HIF-1α, regulated at the level of transcription that is dependent on ERK and AKT signaling pathways, and not protein stability. Additionally, data suggests that OSM leads to a switch from aerobic to glycolytic metabolism as well as an increase in expression of angiogenic factors that is dependent on HIF-1α. Our data also demonstrate that the combination of hypoxia and inflammation, such as found during obesity, causes a synergistic effect on HIF-1α, which causes a synergistic effect on downstream targets, such as GLUT1. Collectively, data presented here demonstrate a mechanistic link between inflammation and hypoxia. As investigations continue, elucidating the effects of HIF-1α by OSM during obesity may contribute to a better understanding of how HIF-1α contributes to both survival and adipocyte dysfunction. This understanding may then lead to therapeutic targets for the treatment of obesity-related insulin resistance and adipocyte dysfunction.

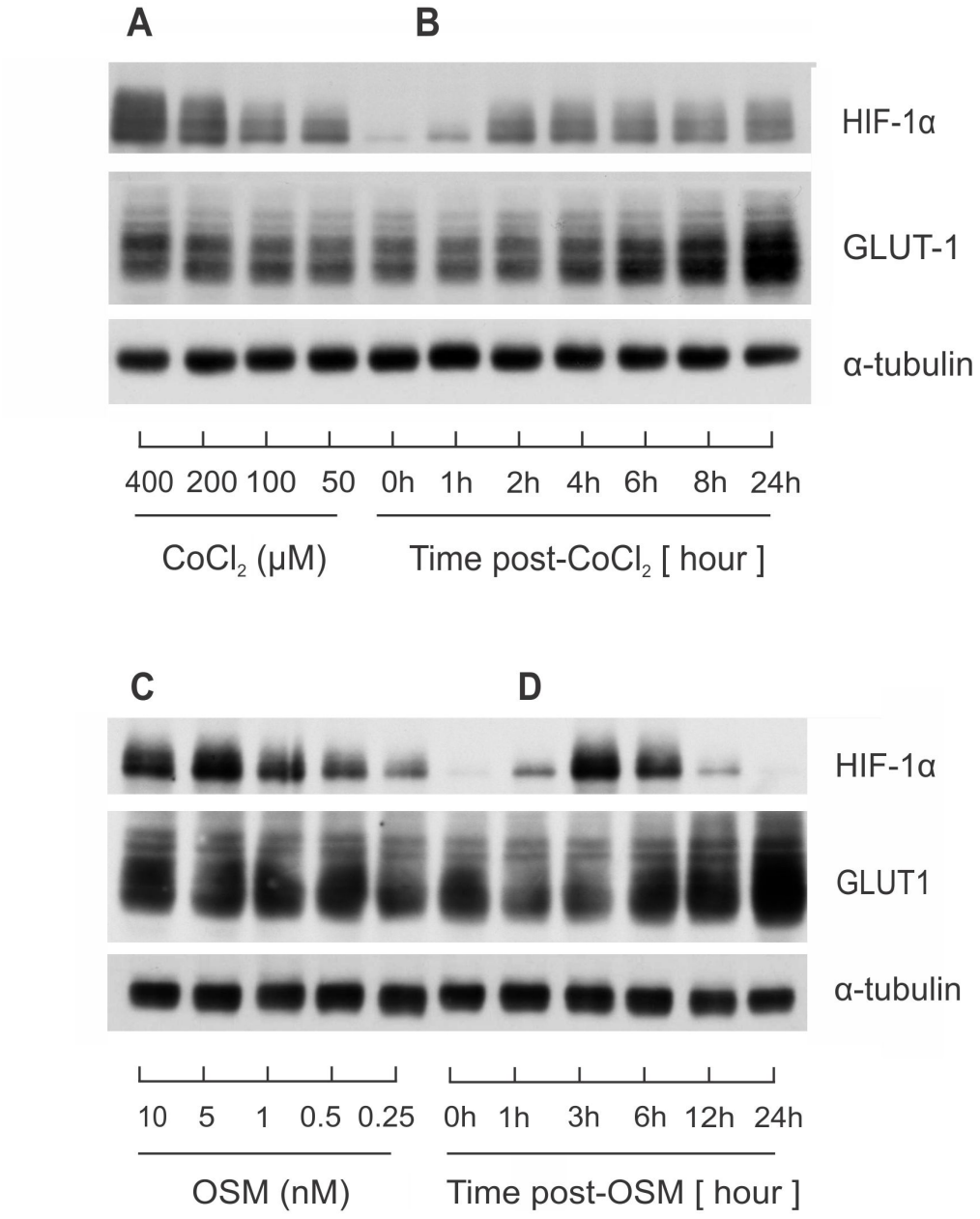

Figure 2.1. **Induction of HIF-1α by $CoCl_2$ and OSM is Dose and Time-Dependent.** Preadipocytes were stimulated with varying doses of $CoCl_2$ and lysates were collected at 6h (A) or with 100μM $CoCl_2$ and collected at the indicated time (B). C) Preadipocytes were stimulated with varying doses of OSM and collected at 6h or stimulated with 1nM OSM and collected over time (D). Cell lysates were analyzed for protein expression of HIF-1α, GLUT1 and α-tubulin via immunoblot analysis.

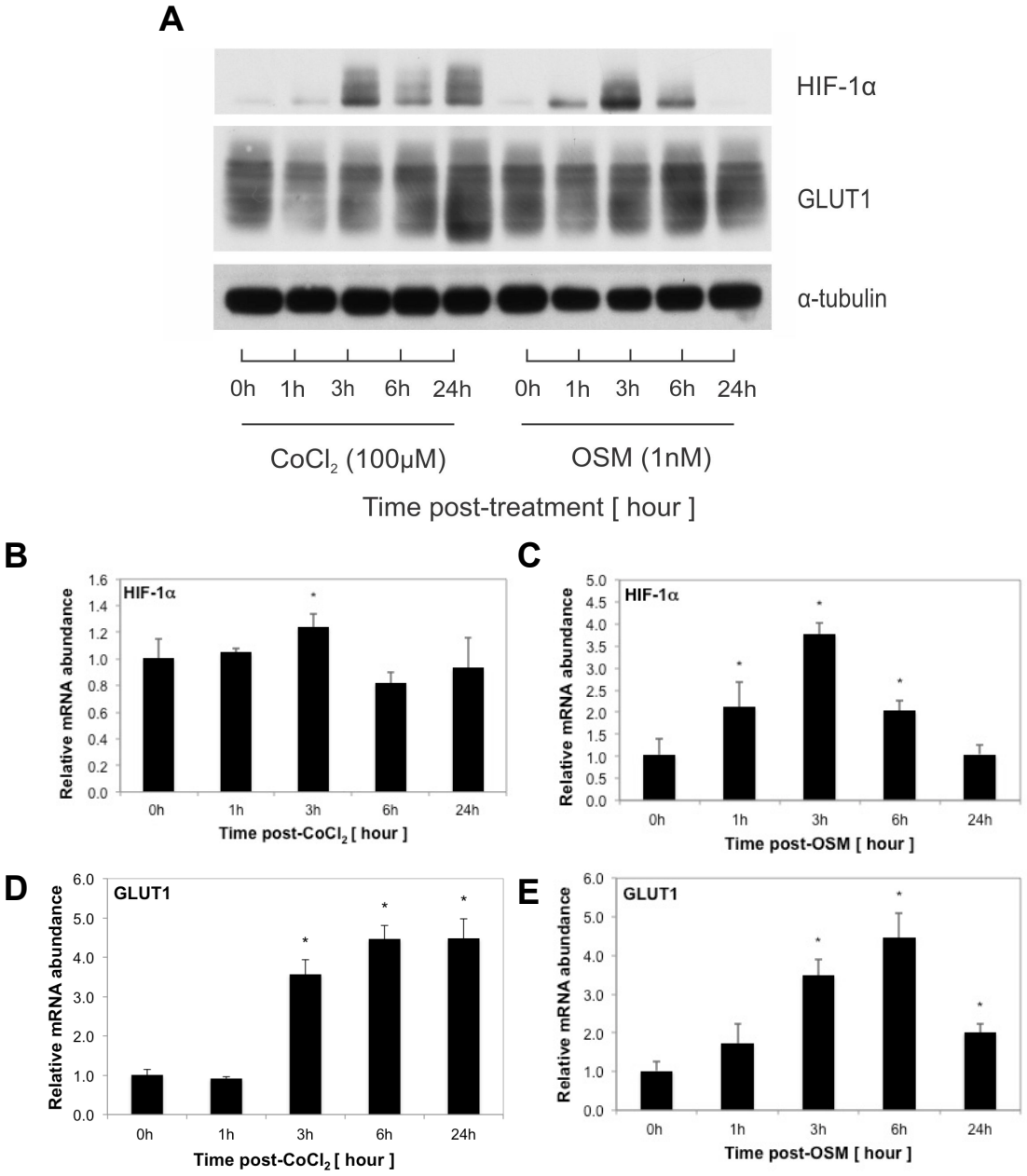

Figure 2.2. OSM Induces HIF-1α Transcription and Protein. Preadipocytes were stimulated with 100μM $CoCl_2$ or 1nM OSM. A) Cell lysates were collected over time and protein expression of HIF-1α and GLUT1 was analyzed via immunoblotting. B) Total RNA was collected and gene expression of HIF-1α and GLUT1 was measured via qRT-PCR. All data were normalized to 18S rRNA and expressed relative to untreated cells (0h). Statistical significance was determined by Student's *t*-test (* $p<0.05$).

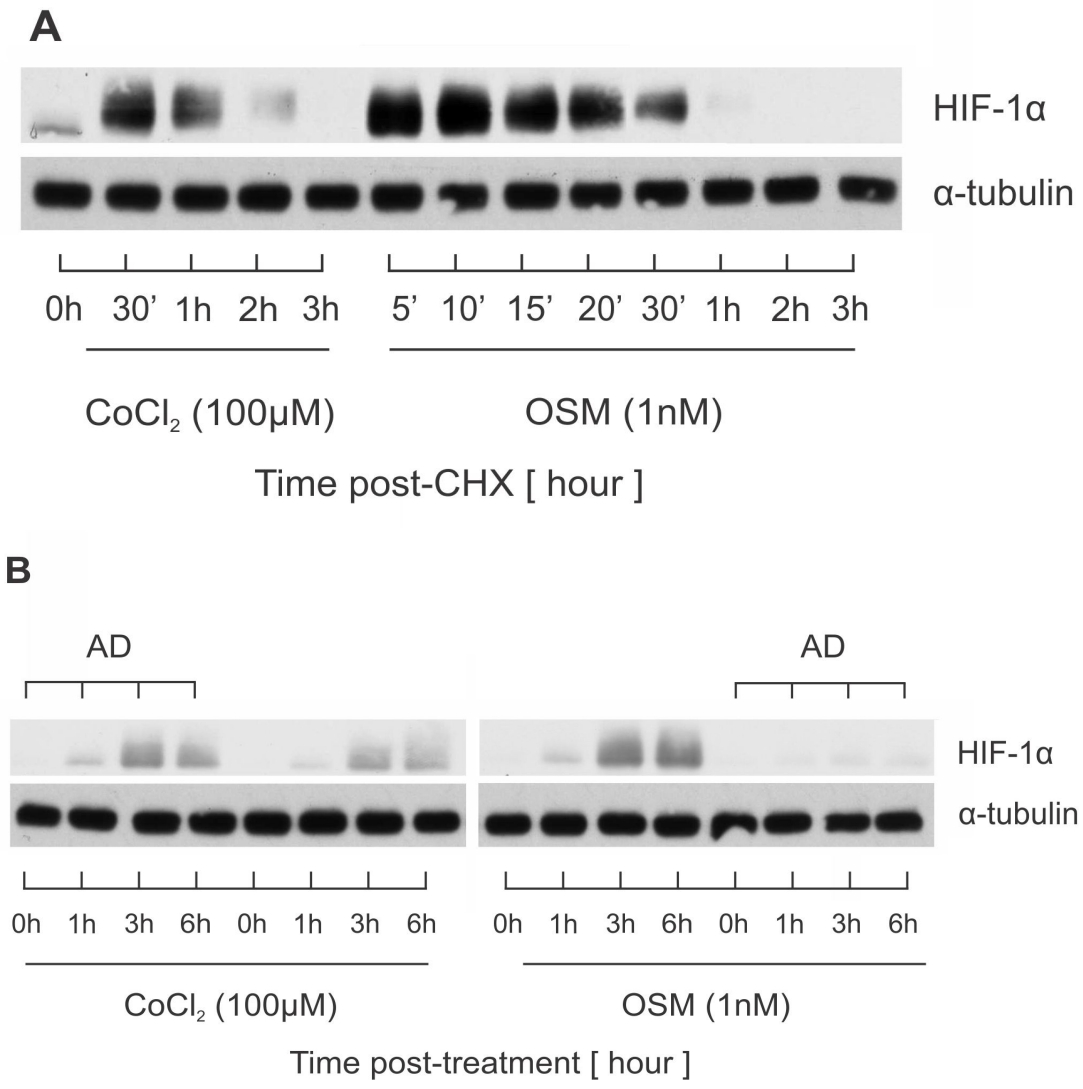

Figure 2.3. OSM-Mediated HIF-1α Up-Regulation is Due to Transcription and not Protein Stability. A) Preadipocytes were pretreated with 100μM $CoCl_2$ or 1nM OSM for 3h before stimulation with 5μM cycloheximide (CHX). Cell lysates were collected over time post-CHX and protein expression of HIF-1α and α-tubulin was analyzed via immunoblotting. B) Preadipocytes were stimulated with either 100μM $CoCl_2$ or 1nM OSM in the absence or presence of 1 ng/ml Actinomycin D (AD). Cell lysates were collected over time post-AD and protein expression of HIF-1α and α-tubulin was analyzed via immunoblotting.

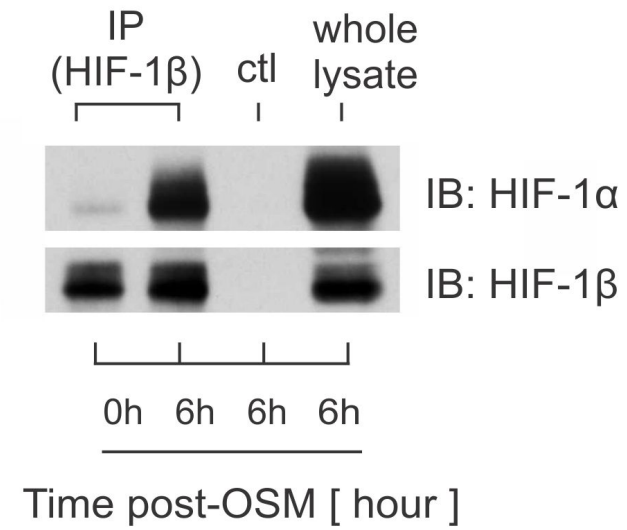

Figure 2.4. OSM-Induced HIF-1α Interacts with HIF-1β to Form Functional Heterodimeric Complex. Cell lysates were harvested from preadipocytes at 0h and 6h post-OSM (1nM), immunoprecipitated for HIF-1β (lane 1 and 2) and immunoblotted for HIF-1α and HIF-1β. Lane 3: control, resin with no antibody attached. Lane 4: whole cell lysates.

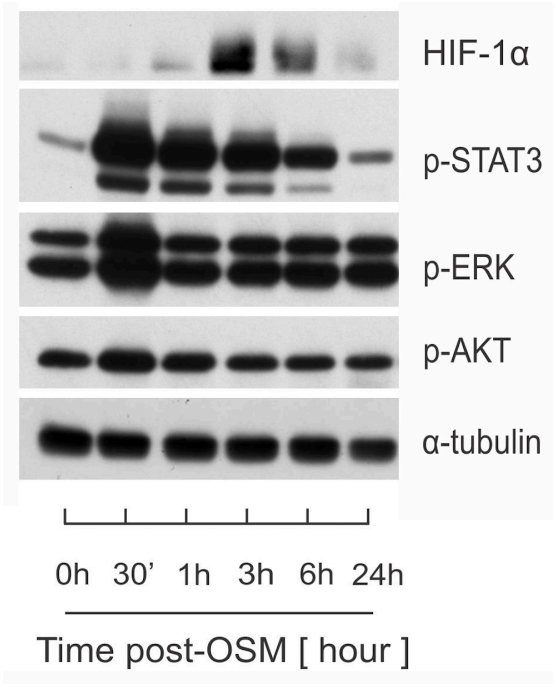

Figure 2.5. OSM Activates STAT, ERK and AKT Signaling Pathways. Preadipocytes were stimulated with 1nM OSM and cell lysates were collected over time. Protein expression of HIF-1α, p-STAT3, p-ERK, p-AKT and α-tubulin was analyzed via immunoblotting.

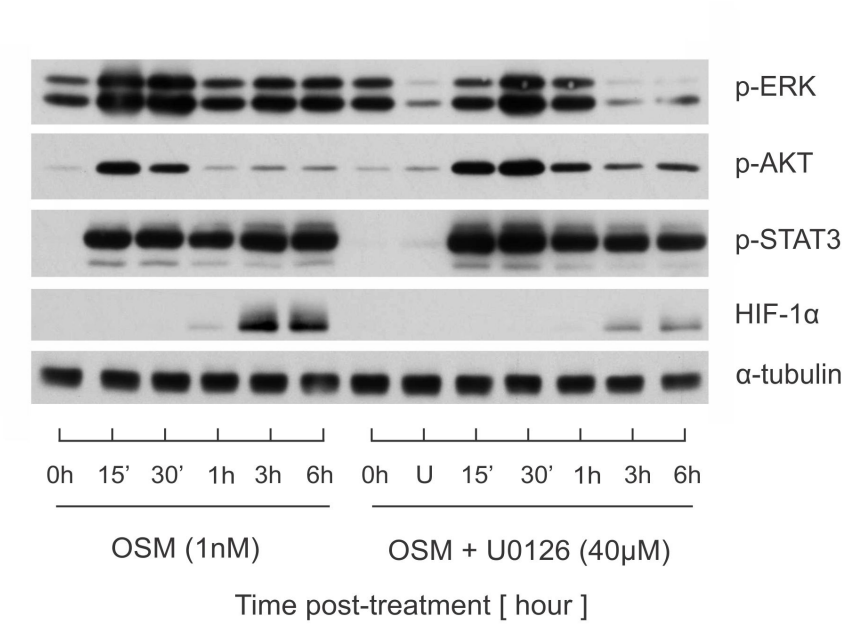

Figure 2.6. OSM-Induced HIF-1α is Dependent on ERK. Preadipocytes were stimulated with 1nM OSM in the absence or presence of 40μM U0126 (1h pretreatment). Cell lysates were harvested at indicated times and immunoblotted for p-ERK, p-AKT, p -STAT3, HIF-1α and α-tubulin.

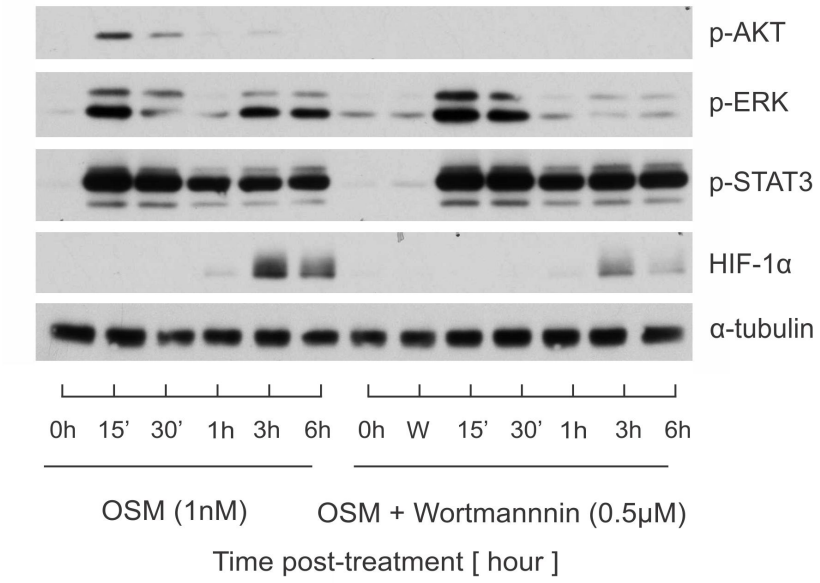

Figure 2.7. OSM-Induced HIF-1α is Dependent on AKT. Preadipocytes were stimulated with 1nM OSM in the absence or presence of 0.5μM Wortmannin (1h pretreatment). Cell lysates were harvested at indicated times and immunoblotted for p-AKT, p-ERK, p-STAT3, HIF-1α and α-tubulin.

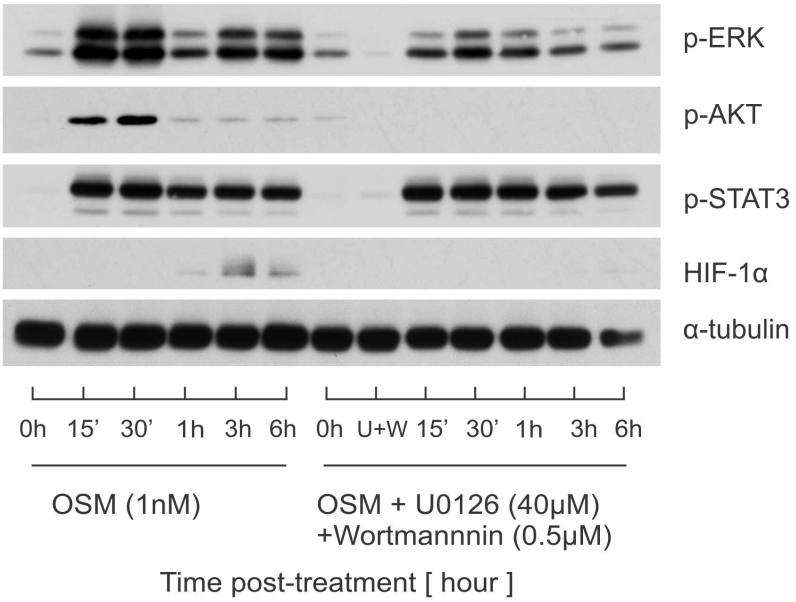

Figure 2.8. OSM-Induced HIF-1α is Dependent on ERK and AKT. Preadipocytes were stimulated with 1nM OSM in the absence or presence of 40μM U0126 and 0.5μM Wortmannin (1h pretreatment). Cell lysates were harvested at indicated times and immunoblotted for p-AKT, p-ERK, p-STAT3, HIF-1α and α-tubulin.

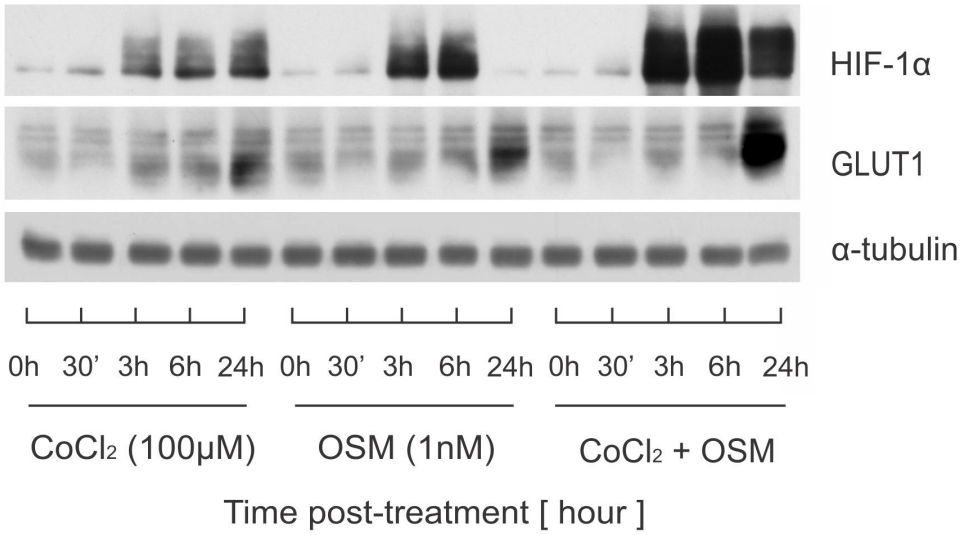

Figure 2.9. $CoCl_2$ and OSM have a Synergistic Effect. Preadipocytes were stimulated with 100μM $CoCl_2$, 1nM OSM or the combination of both $CoCl_2$ and OSM. Cell lysates were collected at the indicated times and immunoblotted for HIF-1α, GLUT1 and α-tubulin.

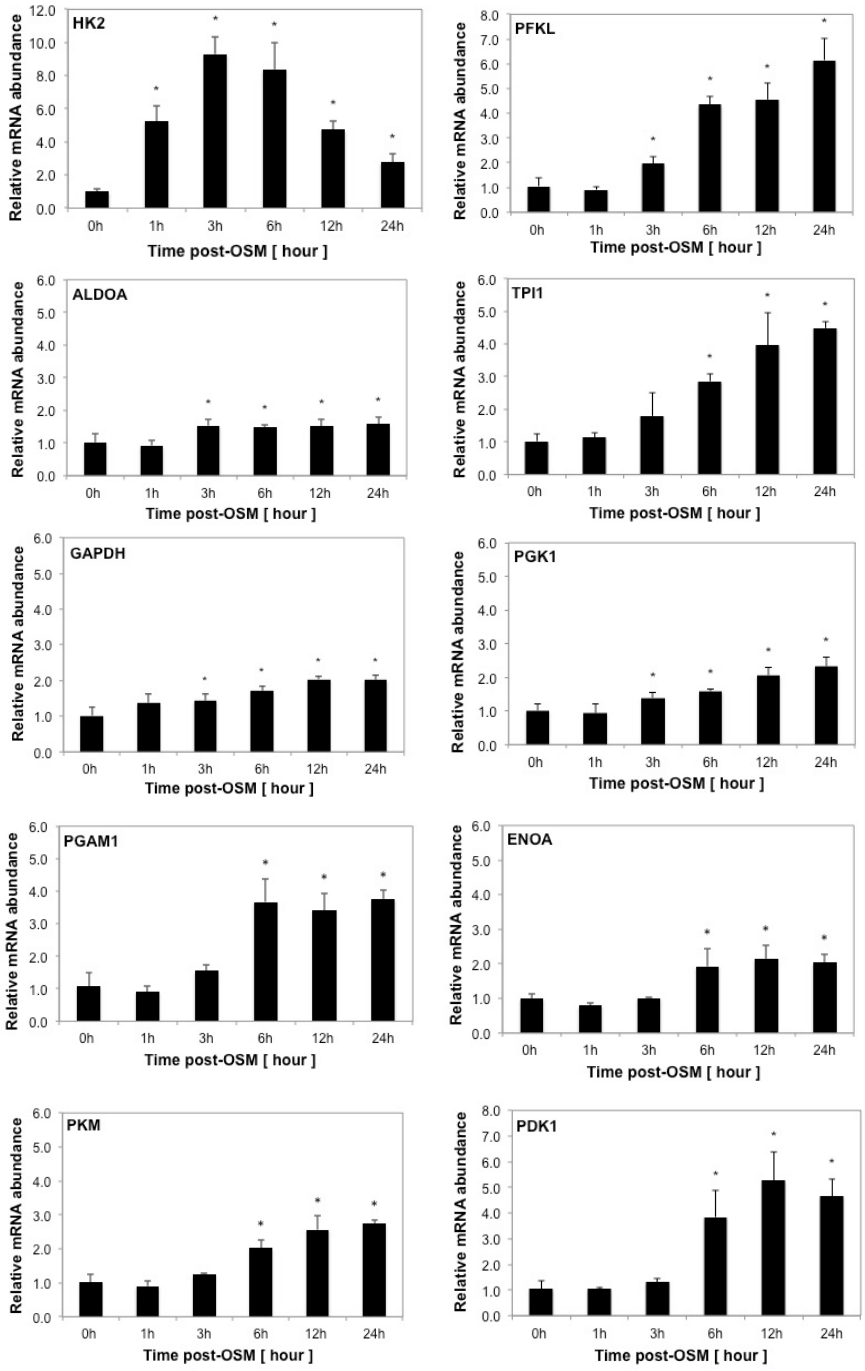

Figure 2.10. OSM-Mediated Up-Regulation of Glycolytic Genes. Preadipocytes were stimulated with 1nM OSM. Cell lysates were collected over time and gene expression of all glycolytic genes and PDK1 was analyzed via qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated PAs (0h). Statistical significance was determined by Student's t-test (* $p<0.05$).

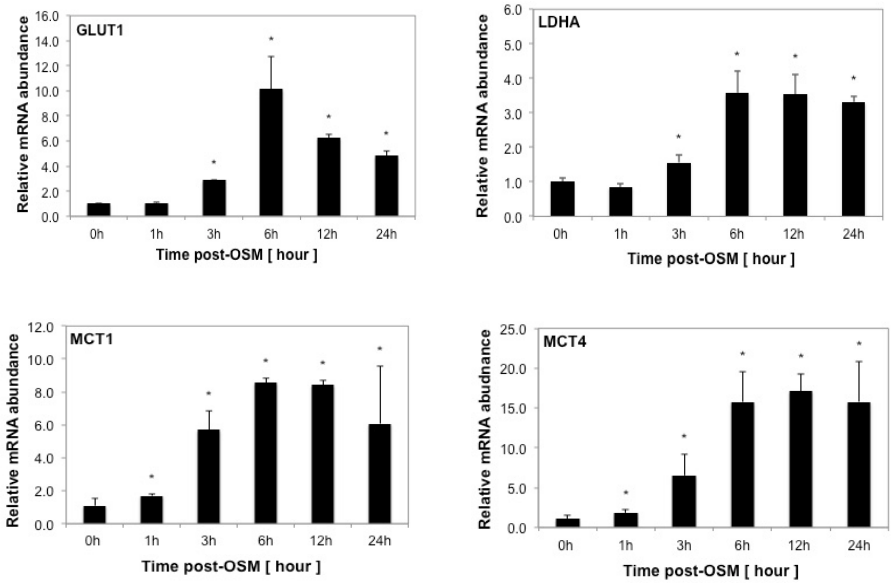

Figure 2.11. OSM Regulates Genes Associated with Glycolysis. Preadipocytes were stimulated with 1nM OSM. Cell lysates were collected over time and gene expression of GLUT1, LDHA, MCT1 and MCT4 was analyzed via qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated PAs (0h). Statistical significance was determined by Student's t-test (* $p<0.05$).

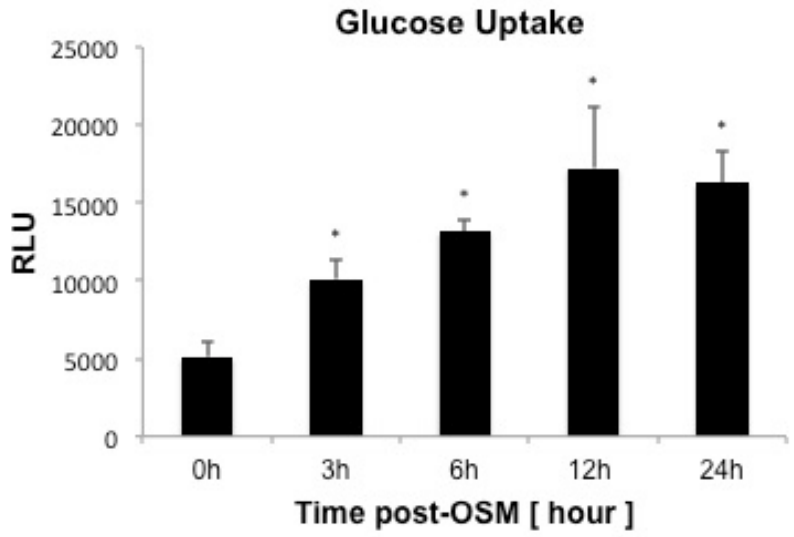

Figure 2.12. OSM Increases Glucose Uptake. Preadipocytes were stimulated with OSM at the indicated times and glucose uptake was measured by luminescence. Statistical significance was determined by Student's t-test (* $p<0.05$).

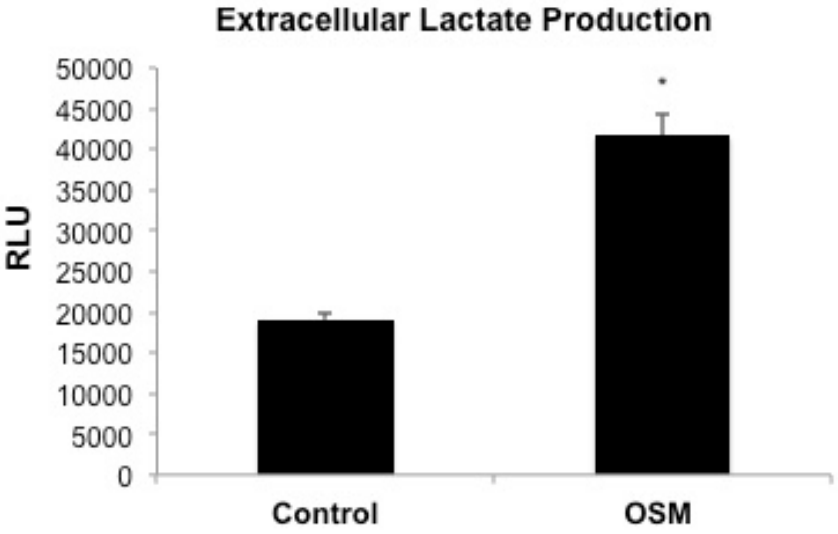

Figure 2.13. OSM Increases Extracellular Lactate Production. Preadipocytes were stimulated with OSM for 24h and extracellular lactate production was measured by luminescence. Statistical significance was determined by Student's t-test (* $p<0.05$).

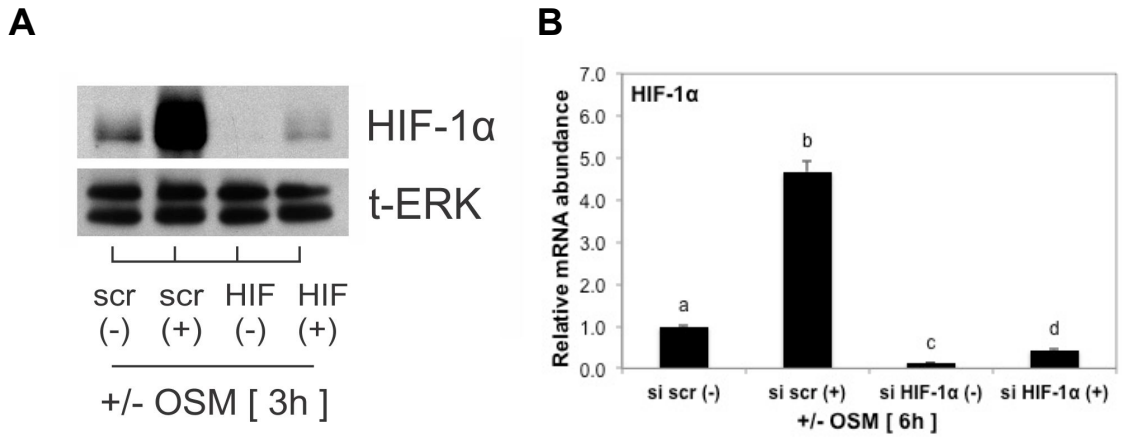

Figure 2.14. Role for HIF-1α in OSM-Mediated Metabolic Adaptation. Preadipocytes were transfected with Lipofectamine RNAiMAX reagent in the presence of non-targeting control siRNA or siRNA for HIF-1α for 48h prior to stimulation with OSM. A) Cell lysates were harvested at 3h post-OSM and analyzed for HIF-1α and t-ERK via immunoblotting. B) Cell lysates were harvested for total RNA and mRNA expression of HIF-1α analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold changes relative to untreated control siRNA (si scr (-)). Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed with the *p* value for the respective parameter was statistically significant (* $p<0.05$).

C

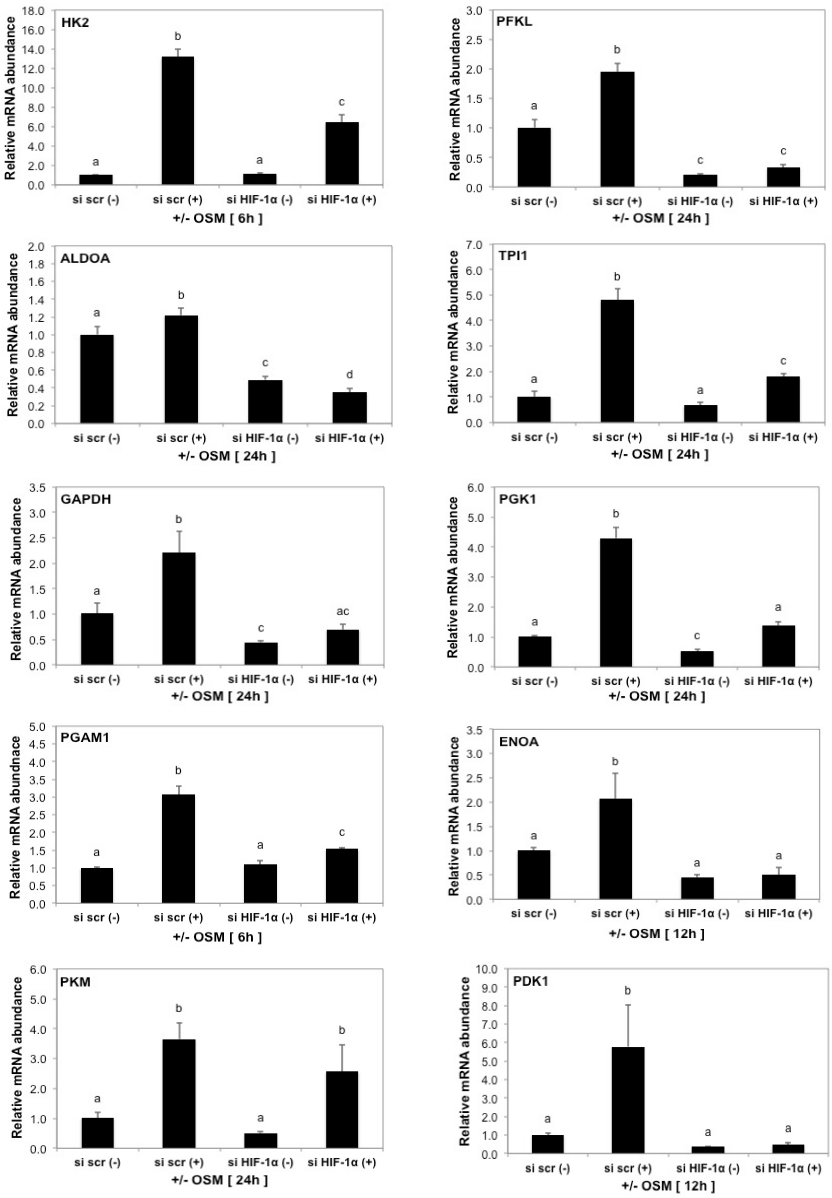

Figure 2.14. Role for HIF-1α in OSM-Mediated Metabolic Adaptation (continued). Preadipocytes were transfected with Lipofectamine RNAiMAX reagent in the presence of non-targeting control siRNA or siRNA for HIF-1α for 48h prior to stimulation with OSM. C) Cell lysates were harvested for total RNA and mRNA expression of all glycolytic genes analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold changes relative to untreated control siRNA (si scr (-)). Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed with the *p* value for the respective parameter was statistically significant ($p<0.05$).

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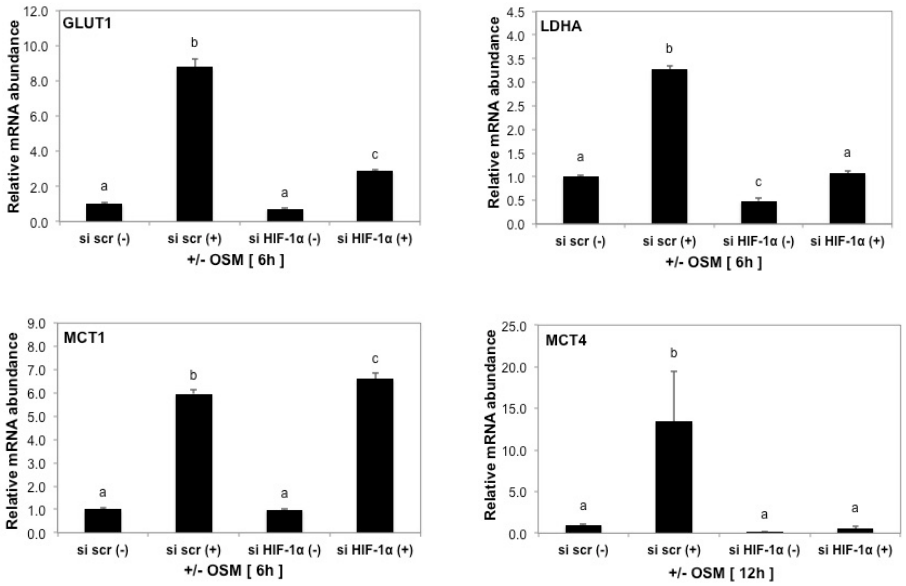


Figure 2.14. Role for HIF-1α in OSM-Mediated Metabolic Adaptation (continued). Preadipocytes were transfected with Lipofectamine RNAiMAX reagent in the presence of non-targeting control siRNA or siRNA for HIF-1α for 48h prior to stimulation with OSM. D) Cell lysates were harvested for total RNA and mRNA expression of GLUT1, LDHA, MCT1 and MCT4 analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold changes relative to untreated control siRNA (si scr (-)). Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed with the *p* value for the respective parameter was statistically significant ($p<0.05$).

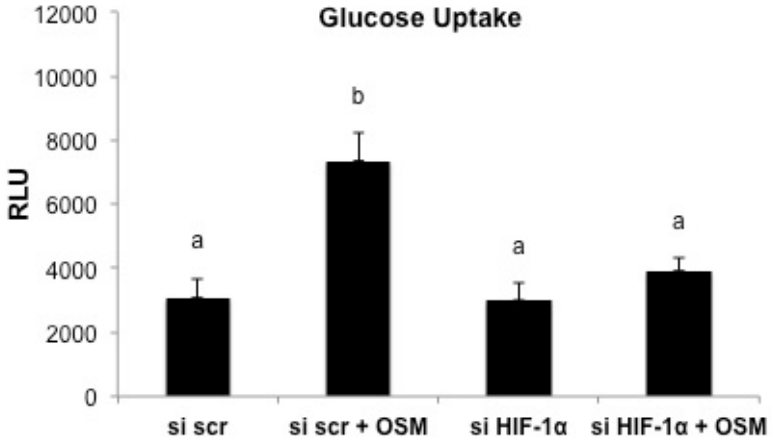


Figure 2.15. Role for HIF-1α in OSM-Mediated Glucose Uptake. Preadipocytes were transfected with Lipofectamine RNAiMAX reagent in the presence of non-targeting control siRNA or siRNA for HIF-1α for 48h prior to stimulation with OSM. Glucose uptake was measured via luminescence at 12h post-OSM. Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed with the *p* value for the respective parameter was statistically significant ($p<0.05$).

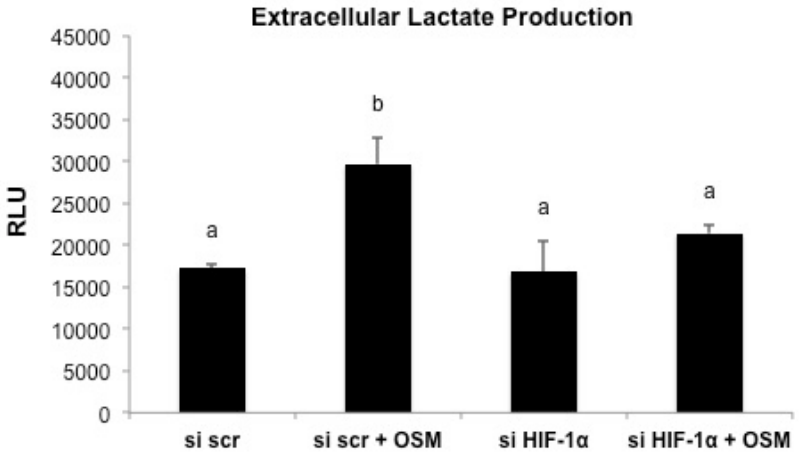


Figure 2.16. Role for HIF-1α in OSM-Mediated Lactate Production. Preadipocytes were transfected with Lipofectamine RNAiMAX reagent in the presence of non-targeting control siRNA or siRNA for HIF-1α for 48h prior to stimulation with OSM. Lactate production was measured via luminescence at 24h post-OSM. Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed with the *p* value for the respective parameter was statistically significant ($p<0.05$).

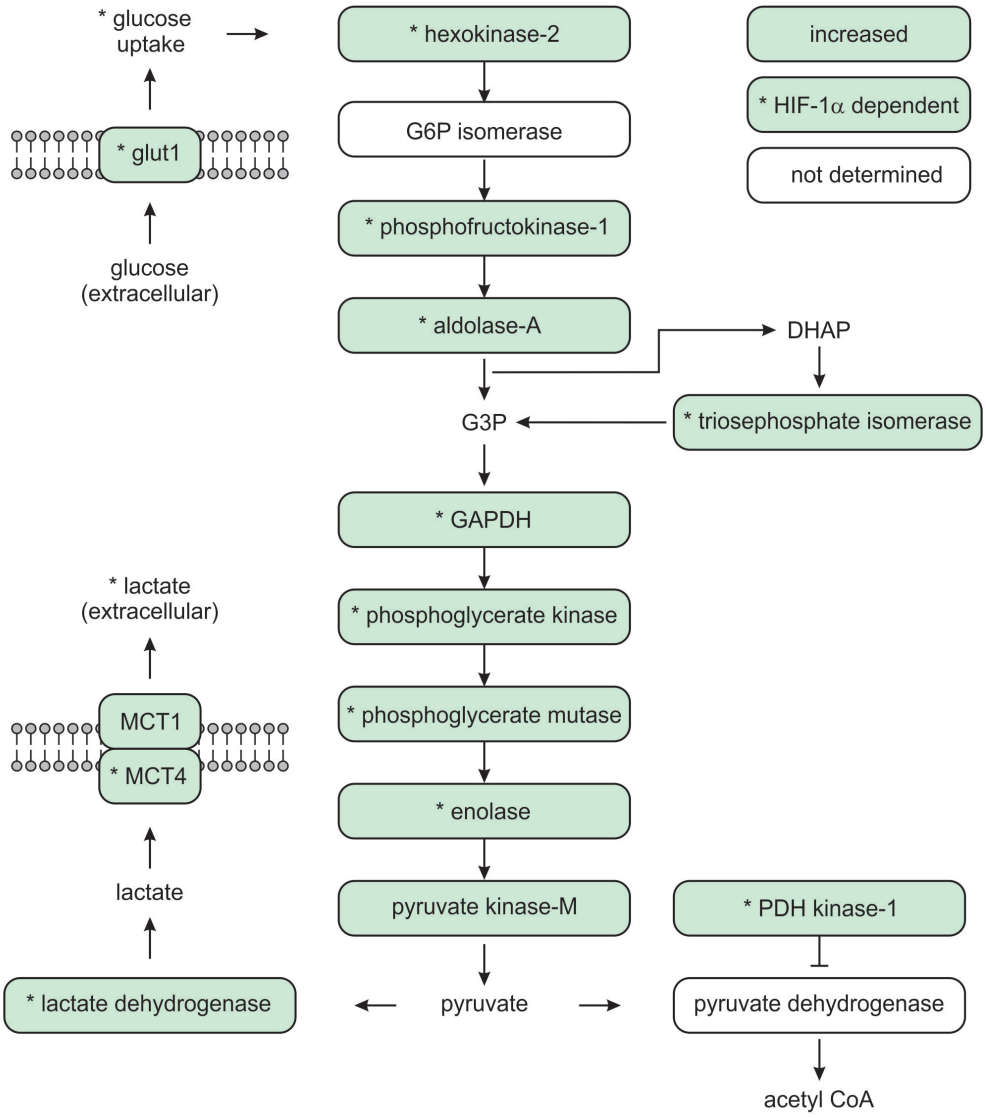


Figure 2.17. Summary of Glycolytic Genes Up-Regulated by OSM and Dependent on HIF-1α.

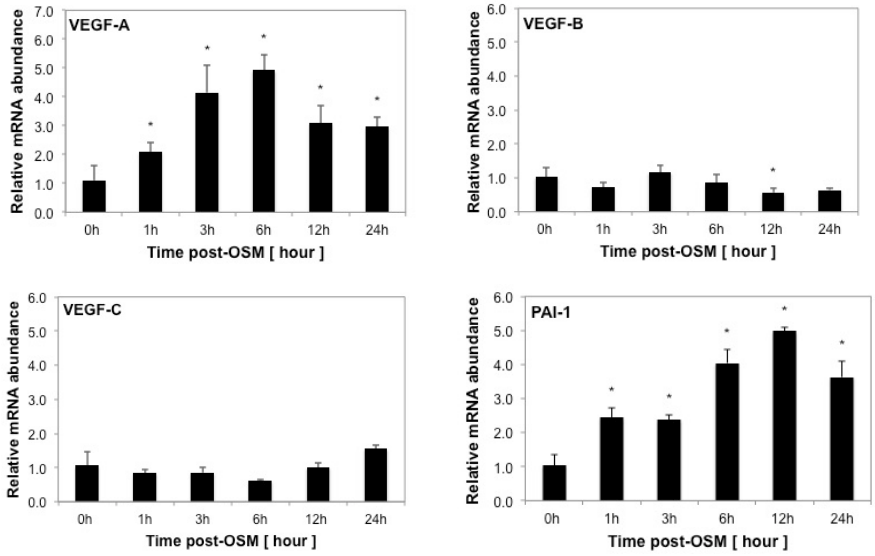


Figure 2.18. OSM Stimulation Increases VEGF and PAI-1. Preadipocytes were stimulated with 1nM OSM. Cell lysates were collected over time and gene expression of VEGFA, VEGFB, VEGFC and PAI-1 was analyzed via qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated PAs (0h). Statistical significance was determined by Student's t-test (* $p<0.05$).

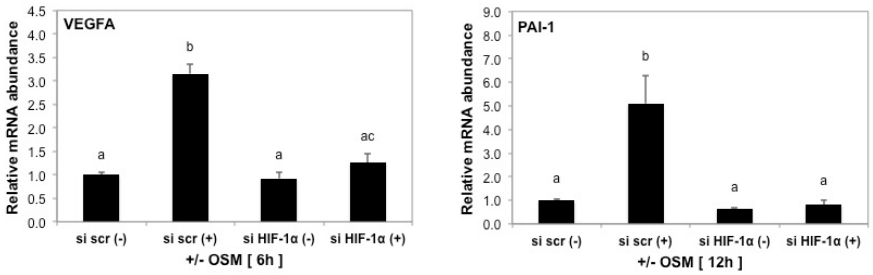


Figure 2.19. Role for HIF-1α in OSM-Induced Expression of VEGF and PAI-1. Preadipocytes were stimulated with 1nM OSM. Cell lysates were collected over time and gene expression of VEGF and PAI-1 was analyzed via qRT-PCR. Data were normalized to 18S rRNA and changes in gene expression measured as fold changes relative to untreated control siRNA (si scr (-)). Statistical differences were determined by ANOVA. Tukey's post-hoc analysis was performed with the *p* value for the respective parameter was statistically significant ($p<0.05$).

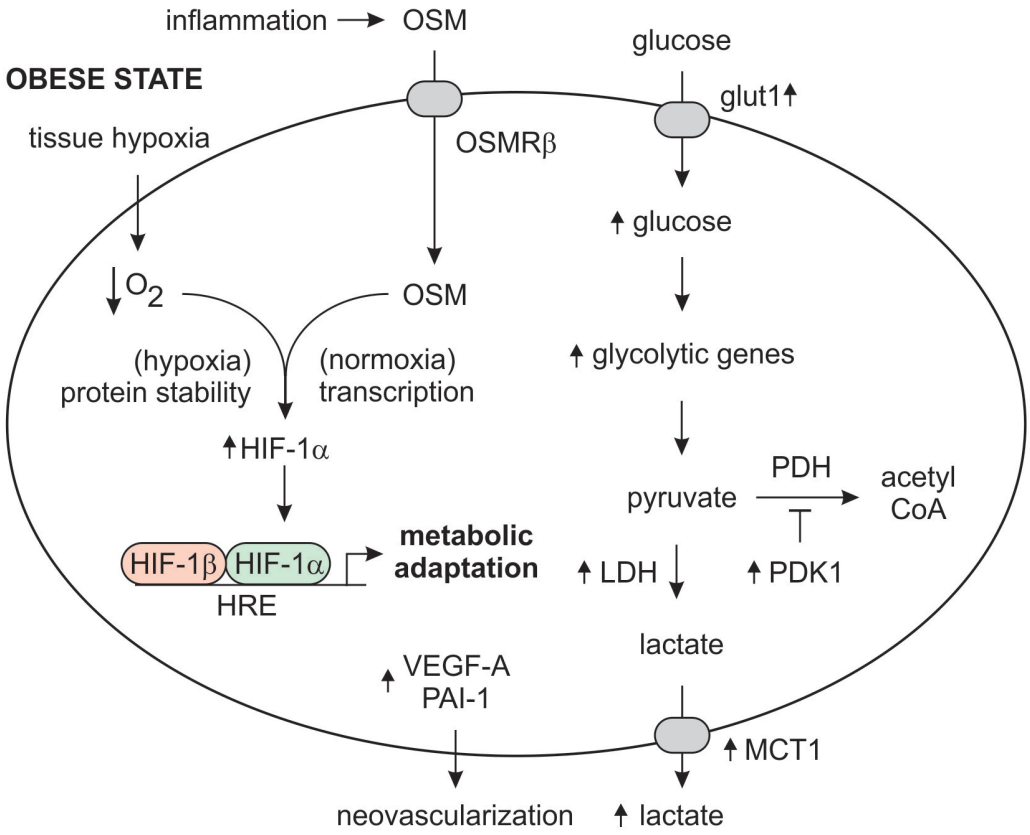


Figure 2.20. Proposed Working Model for Regulation and Function of HIF-1α during Obesity in Response to Hypoxia and OSM in 3T3-L1 Adipocyte Cell Model. During obesity, the combination of tissue hypoxia and increased production of inflammatory marker OSM leads to an increased accumulation of HIF-1α by different mechanisms. Functional HIF-1α then leads to metabolic adaptations within the adipocyte such as increased neovascularization by induction of VEGF-A and PAI-1 and increased glycolytic function through increased glycolytic gene transcription, glucose uptake and lactate production.

Reference List

1. Nguyen T, Lau DCW. The Obesity Epidemic and Its Impact on Hypertension. Can J Cardiol. 2012;28(3):326–33.

2. Barnes AS. The epidemic of obesity and diabetes: trends and treatments. Tex Heart Inst J. 2011;38(2):142–4.

3. Ogden CL, Carroll MD, Fryar CD, Flegal KM. Prevalence of Obesity Among Adults and Youth: United States, 2011-2014. NCHS Data Brief. 2015;(219):1–8.

4. Trayhurn P. Endocrine and signalling role of adipose tissue: New perspectives on fat. Acta Physiol Scand. 2005;184(4):285–93.

5. Rutkowski JM, Stern JH, Scherer PE. The cell biology of fat expansion. Journal of Cell Biology. 2015.

6. Schoettl T, Fischer IP, Ussar S. Heterogeneity of adipose tissue in development and metabolic function. J Exp Biol. 2018;221(jeb162958):1–17.

7. Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB. Adipose tissue remodeling: Its role in energy metabolism and metabolic disorders. Front Endocrinol (Lausanne). 2016;7(APR):1–16.

8. Trayhurn P. Hypoxia and Adipocyte Physiology: Implications for Adipose Tissue Dysfunction in Obesity. Annu Rev Nutr. 2014;34(1):207–36.

9. Duan C. Hypoxia-inducible factor 3 biology: complexities and emerging themes. Am J Physiol Cell Physiol. 2016;310(4):C260–9.

10. Weidemann A, Johnson RS. Biology of HIF-1α. Cell Death Differ. 2008;15(4):621–7.

11. Jaakkola P, Mole DR, Tian Y, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of HIF-alpha to the von Hippel-Lindau Ubiquitylation Complex by Oxygen-Regulated Prolyl Hydroxylation. Science (80-). 2001;292(5516):468–72.

12. Dengler VL, Galbraith MD, Espinosa JM. Transcriptional regulation by hypoxia inducible factors. Crit Rev Biochem Mol Biol. 2014;49(1):1–15.

13. Eales KL, Hollinshead KER, Tennant DA. Hypoxia and metabolic adaptation of cancer cells. Oncogenesis. 2016;5(1):e190.

14. Kim JW, Tchernyshyov I, Semenza GL, Dang C V. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. Cell Metab. 2006;3(3):177–85.

15. Wang B, Wood IS, Trayhurn P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. Pflugers Arch Eur J Physiol. 2007;455(3):479–92.

16. Park HS, Kim J, Sun B, Song S, Suh W, Sung J. Hypoxia induces glucose uptake and metabolism of adipose-derived stem cells. Mol Med Rep. 2016;4706–14.

17. Geiger K, Leiherer A, Muendlein A, Stark N, Geller-Rhomberg S, Saely CH, et al. Identification of hypoxia-induced genes in human SGBS adipocytes by microarray analysis. PLoS One. 2011;6(10).

18. Greijer AE, van der Groep P, Kemming D, Shvarts A, Semenza GL, Meijer GA, et al. Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor I (HIF-1). J Pathol. 2005;206(3):291–304.

19. Mazzatti D, Lim F-L, O'Hara A, Wood IS, Trayhurn P. A microarray analysis of the hypoxia-induced modulation of gene expression in human adipocytes. Arch Physiol Biochem. 2012;118(3):112–20.

20. Wood IS, De Heredia FP, Wang B, Trayhurn P. Cellular hypoxia and adipose tissue dysfunction in obesity. Proc Nutr Soc. 2009;68(4):370–7.

21. Wood IS, Stezhka T, Trayhurn P. Modulation of adipokine production , glucose uptake and lactate release in human adipocytes by small changes in oxygen tension. Eur J Physiol. 2011;469–77.

22. Hajer GR, Van Haeften TW, Visseren FLJ. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J. 2008;29(24):2959–71.

23. Hahn WS, Kuzmicic J, Burrill JS, Donoghue MA, Foncea R, Jensen MD, et al. Proinflammatory cytokines differentially regulate adipocyte mitochondrial metabolism, oxidative stress, and dynamics. AJP Endocrinol Metab. 2014;306(9):E1033–45.

24. Hotamisligil GS, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest. 1995;95(5):2409–15.

25. Chen XH, Zhao YP, Xue M, Ji CB, Gao CL, Zhu JG, et al. TNF-α induces mitochondrial dysfunction in 3T3-L1 adipocytes. Mol Cell Endocrinol. 2010;328(1–2):63–9.

26. Sanchez-Infantes D, White UA, Elks CM, Morrison RF, Gimble JM, Considine R V., et al. Oncostatin M is produced in adipose tissue and is regulated in conditions of obesity and type 2 diabetes. J Clin Endocrinol Metab. 2014;99(2):217–25.

27. Elks CM, Zha P, Grant RW, Hang H, Bailey JL, Burk DH, et al. Loss of Oncostatin M Signaling in Adipocytes Induces Insulin Resistance and Adipose Tissue Inflammation in Vivo*. J Biol Chem. 2016;291(33):17066–76.

28. Rega G, Kaun C, Weiss TW, Demyanets S, Zorn G, Kastl SP, et al. Inflammatory cytokines interleukin-6 and oncostatin M induce plasminogen activator inhibitor-1 in human adipose tissue. Circulation. 2005;111(15):1938–45.

29. Rega G, Kaun C, Demyanets S, Pfaffenberger S, Rychli K, Hohensinner PJ, et al. Vascular endothelial growth factor is induced by the inflammatory cytokines interleukin- 6 and oncostatin m in human adipose tissue in vitro and in murine adipose tissue in vivo. Arterioscler Thromb Vasc Biol. 2007;27(7):1587–95.

30. Haddad JJ, Harb HL. Cytokines and the regulation of hypoxia-inducible factor (HIF)-1 alpha. Int Immunopharmacol. 2005;5:461–83.

31. Scharte M, Han X, Bertges DJ, Fink MP, Delude RL, Han X, et al. Cytokines induce HIF-1 DNA binding and the expression of HIF-1-dependent genes in cultured rat enterocytes. Am J Physiol Gastrointest Liver Physiol. 2018;284:G373–84.

32. Vollmer S, Kappler V, Kaczor J, Flügel D, Rolvering C, Kato N, et al. Hypoxia-inducible factor 1α is up-regulated by oncostatin M and participates in oncostatin M signaling. Hepatology. 2009;50(1):253–60.

33. Battello N, Zimmer AD, Goebel C, Dong X, Behrmann I, Haan C, et al. The role of HIF-1 in oncostatin M-dependent metabolic reprogramming of hepatic cells. Cancer Metab. 2016;4(1):3.

34. Ferguson BS, Nam H, Hopkins RG, Morrison RF. Impact of reference gene selection for target gene normalization on experimental outcome using real- time qRT-PCR in adipocytes. PLoS One. 2010;5(12).

35. Livak Kenneth J STD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T))Method. Methods. 2001;25(4):402–8.

36. Jung Y, Isaacs JS, Lee S, Trepel J, Liu Z-G, Neckers L. Hypoxia-inducible factor induction by tumour necrosis factor in normoxic cells requires receptor-interacting protein-dependent nuclear factor kappa B activation. Biochem J. 2003;370(Pt 3):1011–7.

37. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-Inducible Factors and the Response to Hypoxic Stress. Mol Cell. 2010;40(2):294–309.

38. Richard DE, Berra E, Pouysségur J. Non-hypoxic pathway mediates the induction of hypoxia inducible factor 1 alpha (HIF-1α) in vascular smooth muscle. J Biol Chem. 2000;275(35):26765–71.

39. Isaacs JS, Jung YJ, Neckers L. Aryl Hydrocarbon Nuclear Translocator (ARNT) Promotes Oxygen-independent Stabilization of Hypoxia-inducible Factor-1α by Modulating an Hsp90-dependent Regulatory Pathway. J Biol Chem. 2004;279(16):16128–35.

40. Richards CD. The Enigmatic Cytokine Oncostatin M and Roles in Disease. ISRN Inflamm. 2013;512103.

41. Hermanns HM. Oncostatin M and interleukin-31: Cytokines, receptors, signal transduction and physiology. Cytokine Growth Factor Rev. 2015;26(5):545–58.

42. Miyaoka Y, Tanaka M, Naiki T, Miyajima A. Oncostatin M inhibits adipogenesis through the RAS/ERK and STAT5 signaling pathways. J Biol Chem. 2006;281(49):37913–20.

43. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 2003;3(10):721–32.

44. Lin Q, Lee YJ, Yun Z. Differentiation arrest by hypoxia. J Biol Chem. 2006;281(41):30678–83.

45. Elks CM, Stephens JM. Oncostatin m modulation of lipid storage. Biology (Basel). 2015;4(1):151–60.

46. Semenza GL. Hypoxia-Inducible Factor 1: Regulator of Mitochondrial Metabolism and Mediator of Ischemic Preconditioning. Biochim Biophys Acta. 2012;1813(7):1263–8.

47. Hotta K, Funahashi T, Bodkin NL, Ortmeyer HK, Arita Y, Hansen BC, et al. Circulating concentrations of the adipocyte protein adponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in Rhesys Monkeys. Diabetes. 2001;50(May):1126–33.

48. Isogai C, Laug WE, Shimada H, Declerck PJ, Stins MF, Durden DL, et al. Plasminogen Activator Inhibitor-1 Promotes Angiogenesis by Stimulating Endothelial Cell Migration toward Fibronectin. Cancer Res. 2001;61:5587–94.

49. Corvera S, Gealekman O. Adipose Tissue Angiogenesis: Impact on Obesity and Type-2 Diabetes. Biochim Biophys Acta. 2015;1842(3):463–72.

50. Elias I, Franckhauser S, Bosch F. New insights into adipose tissue VEGF-A actions in the control of obesity and insulin resistance. Adipocyte. 2013;2(June):109–12.

51. Sun K, Wernstedt I, Kusminski CM, Carolina A, Wang Z V, Pollard JW, et al. Dichotomous effects of VEGF-A on adipose tissue dysfunction. Proc Natl Acad Sci. 2012;

52. Li X, Lee C, Tang Z, Zhang F, Arjunan P, Li Y, et al. VEGF-B: A survival, or an angiogenic factor? Cell Adhes Migr. 2009;3(4):322–7.

53. AlessI MC, Poggi M, Juhan-Vague I. Plasminogen activator inhibitor-1, adipose tissue and insulin resistance. CurrOpinLipidol. 2007;18(0957–9672 (Print)):240–5.

54. Juhan-Vague I, Alessi MC, Mavri A, Morange PE. Plasminogen activator inhibitor-1, inflammation, obesity, insulin resistance and vascular risk. J Thromb Haemost. 2003;1(7):1575–9.

55. Lolmède K, Durand de Saint Front V, Galitzky J, Lafontan M, Bouloumié a. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. Int J Obes Relat Metab Disord. 2003;27(10):1187–95.

56. Klöting N, Blüher M. Adipocyte dysfunction, inflammation and metabolic syndrome. Rev Endocr Metab Disord. 2014;15(4):277–87.

57. Laforest S, Labrecque J, Michaud A, Cianflone K, Tchernof A. Adipocyte size as a determinant of metabolic disease and adipose tissue dysfunction. Crit Rev Clin Lab Sci. 2015;52(6):301–13.

58. Karpe F, Fielding B a, Ilic V, Macdonald I a, Summers LKM, Frayn KN. Response Is Related to Aspects of Insulin Sensitivity. Diabetes. 2002;2(18):2467–73.

59. Sun K, Halberg N, Khan M, Magalang UJ, Scherer PE. Selective Inhibition of Hypoxia-Inducible Factor 1 Ameliorates Adipose Tissue Dysfunction. Mol Cell Biol. 2013;33(5):904–17.

60. Pasarica M, Rood J, Ravussin E, Schwarz JM, Smith SR, Redman LM. Reduced oxygenation in human obese adipose tissue is associated with impaired insulin suppression of lipolysis. J Clin Endocrinol Metab. 2010;95(8):4052–5.

61. Wood IS, Wang B, Lorente-Cebrián S, Trayhurn P. Hypoxia increases expression of selective facilitative glucose transporters (GLUT) and 2-deoxy-d-glucose uptake in human adipocytes. Biochem Biophys Res Commun. 2007;361(2):468–73.

62. Wang B, Wood IS, Trayhurn P. PCR arrays identify metallothionein-3 as a highly hypoxia-inducible gene in human adipocytes. Biochem Biophys Res Commun. 2008;368(1):88–93.

63. Docanto, M, Ham S, Corbould A, Brown K. Obesity-Associated Inflammatory Cytokines and Prostaglandin E2 Stimulate Glucose Transporter mRNA Expression and Glucose Uptake in Primary Human Adipose Stromal Cells. J Interf Cytokine Res. 2015;35(8):600–5.

CHAPTER III

ROLE FOR HIF-1α IN THE EFFECT OF OSM ON ADIPOCYTE GROWTH AND DIFFERENTIATION

Abstract

Knowledge concerning the regulation of proliferation and differentiation (collectively termed hyperplasia) of preadipocytes (PAs) during obesity is critical for understanding adipose tissue remodeling and its related function. A growing body of evidence indicates that the local hypoxia and cytokine secretion accompanying obesity affects adipose tissue hyperplasia. Using proliferating and differentiating 3T3-L1 PAs, this study examined the impact of the inflammatory cytokine, Oncostatin M (OSM) on adipocyte cell growth and differentiation. Data presented here demonstrate that OSM inhibited proliferation of subconfluent PAs while concurrently reducing cyclin D1, cyclin A, cyclin B1 and p-RB, suggesting OSM affects cell growth by a mechanism related to cell cycle. We further demonstrate that OSM inhibited differentiation during mitotic clonal expansion (MCE) by inhibiting PPARγ (peroxisome proliferator-activated receptor γ), C/EBPα (CCAAT/enhancer-binding protein α), aP2 (adipocyte protein 2) and ADPN (adiponectin). Additionally, OSM dramatically reduced cyclin D1 during differentiation. Moreover, we show that OSM induces hypoxia inducible factor-1α (HIF-1α) during differentiation and that HIF-1α-specific knockdown partially restores PPARγ, C/EBPα, aP2 and cyclin D1. Collectively, these results suggest that HIF-1α plays a mechanistic role in the OSM-mediated effects on cell growth and differentiation during normoxia.

Introduction

According to the World Health Organization (WHO), between 1975 and 2016, the prevalence of obesity worldwide tripled, leading to increased obesity-related complications such as cardiovascular disease, diabetes and cancer. The Centers for Disease Control Prevention (CDC) estimates that the prevalence of obesity in the US alone in 2015~2016 was 39.8%, totaling about 93.3 million affected US adults. It is widely accepted that the development of obesity-related diseases is, in part, regulated by white adipose tissue (WAT), which serves not only as an energy reservoir, but as a main secretory organ and site of inflammation. In fact, insulin resistance and metabolic syndrome have been directly linked to inflammatory cytokine secretions (1). Cytokines are involved in a variety of biological process such as proliferation, differentiation, mitochondrial metabolism and insulin resistance (2,3).

Obesity can be classified into two main categories of hyperplasia (increase in adipocyte number) and hypertrophy (increase in adipocyte size) (4). These mechanisms contribute to adipose tissue remodeling, which includes alterations in the number and size of adipocytes during obesity (5). The resulting hyperplasia occurs through both the proliferation of preadipocytes (PAs) and adipocyte differentiation of PAs to mature adipocytes (ADs) (6–8). Specifically, mitotic clonal expansion (MCE), which occurs during early phases of differentiation, mimics PA hyperplasia by requiring cells to re-enter cell cycle and go through post-confluent mitosis (4,7). Cell cycle is a tightly regulated process with four different phases: Gap 1 (G1), synthesis (S), Gap 2 (G2) and mitosis (M), which are each associated with regulatory cyclin proteins (9) (Fig.3.1A). Cell cycle progression is highly regulated by cyclin dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs) (10). G1 is characterized by cell growth to prepare for

DNA replication and requires CDK4 and CDK6 to be coupled with cyclin D1 to promote passage through G1 phase by the inactivation of RB by phosphorylation (10,11). This inactivation of RB causes the transcription factor, E2F to be released so that it can transcribe S-phase genes. Thus, cyclin D1 plays an obligatory role in regulating G1 progression. This progression is a critical step as once cells enter S phase, they are committed to cell division (9,11). Additionally, cyclin D1 has been shown to regulate differentiation, independent of cell cycle progression (12,13). As illustrated in Fig.3.1A, adipocyte differentiation, or adipogenesis is characterized by sequential increases in critical adipogenic proteins such as C/EBPβ, C/EBPδ, C/EBPα, PPARγ, aP2 and ADPN (14). Specifically, PPARγ is considered the "master regulator" of adipogenesis and requires C/EBPβ for its induction (15–17) (Fig.3.1B). These transcription factors allow for the activation of genes that create the phenotype of adipocytes (16).

Oncostatin M (OSM) is a gp130 or IL-6 type cytokine originally discovered based on its antiproliferative activity in a human melanoma cell line (18). Among the various biological processes that gp130 cytokines are involved in, these cytokines have recently been studied as potential therapeutic targets in obesity as research has shown that adipocytes and adipose tissue are sensitive to their secretion. OSM is unique because it has it's own specific receptor that heterodimerizes with gp130 termed OSMRβ (19). While OSM is not specifically produced by PAs or ADs, both cell types have OSM receptors, allowing them to be responsive to OSM secreted from other cells within the adipose tissue such as T-cells and macrophages (20,21). Importantly, both OSM and OSMRβ levels are significantly increased during conditions of obesity (22,23). It is well accepted that the increases in OSM inhibit adipocyte differentiation (3,21,24–26). Additionally, in several cell types, OSM regulates cellular proliferation, as well as cell

cycle progression by inhibiting the transition from G1 to S phase and regulating cyclin D1 protein (19,20,27). Our lab, as well as others, has shown that OSM up-regulates hypoxia-inducible factor-1 alpha (HIF-1α) (28). HIF-1α is normally regulated under low oxygen conditions, which occurs within the adipose tissue as it expands with obesity (29). Like OSM, hypoxia also inhibits differentiation and regulates cell cycle progression (30–33). Specifically, hypoxia-mediated inhibition of adipogenesis has been shown to involve a direct effect of HIF-1α on PPARγ (34). Collectively, increases in both OSM and hypoxia occur during obesity and cause changes in cell growth, differentiation and cell cycle progression. However, whether the effects of OSM on differentiation and cell cycle are due to HIF-1α is unknown. Hence, this study investigated the effect of OSM on adipocyte growth and differentiation and the role of HIF-1α in these changes.

In this report, we examined the impact of OSM on adipose tissue remodeling by examining both PA growth and AD differentiation using 3T3-L1 murine adipocytes. This cell line allowed for the examination of both PA proliferation as well as differentiation from PAs to mature ADs. This study has determined that OSM inhibits both PA proliferation, as well as differentiation. Data reveal that OSM reduces cyclin D1 in both subconfluent proliferating PAs and during the proliferative MCE phase of differentiation. These data suggest that OSM causes cells to drop out of cell cycle very early. Due to previous research that connects OSM with HIF-1α, we sought to determine if there is a relationship with HIF-1α and the effect of OSM on cell growth and differentiation. Interestingly, knockdown of HIF-1α partially restored the OSM-mediated inhibition of cyclin D1 and differentiation markers PPARγ, aP2 and C/EPBα. This demonstrates a role for HIF-1α in the effect of OSM on adipogenesis. Collectively, these observations suggest that OSM is not only involved in adipose tissue remodeling via impacts on

proliferation and differentiation, but that HIF-1α is plays a mechanistic role in this process.

Materials and Methods

Materials. Modified Eagle's Medium (DMEM), calf bovine serum (CS) and Trypsin-EDTA were purchased from Invitrogen. Murine Oncostatin M (OSM) was purchased for Sigma. Antibodies used for immunoblotting include cyclin D1, p-RB, ADPN, HIF-1α, HIF-1β, total-ERK and α-tubulin purchased from Cell Signaling, cyclin A, cyclin B1, C/EBPβ, PPARγ, aP2, and C/EBPα were purchased from Santa Cruz Biotechnology and OSMRβ was purchased from R&D Systems. Dicer-substrate siRNA duplexes were purchased from Integrated DNA Technologies. Enhanced chemiluminescence (ECL) reagents were obtained from Perkin-Elmer Life Sciences. All TaqMan primer probes used in this study were purchased from Applied Biosystems.

Cell Culture and Induction of Differentiation. The murine 3T3-L1 cell line was purchased from Howard Green, Harvard Medical School. For proliferation studies, cells were propagated in DMEM supplemented with 10% CS and experiments were conducted in subconfluent proliferating PAs. For differentiation studies growth medium was replaced at 2-days post-confluence with DMEM supplemented with 10% FBS, 0.5mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). Throughout the study, 'time 0' refers to density-arrested cells immediately before the addition of MDI to the culture medium. Throughout the study, 'd0' refers to density-arrested cells immediately prior to MDI stimulation as described in figure legend. Experiments described herein were conducted within the period of differentiation spanning from density arrest (d0) through 8 days (d8) post-MDI. All experiments were repeated at least 3 times to validate results and ensure reliability.

Immunoblotting. Cell monolayers were washed with phosphate-buffer saline (PBS) and scraped into ice-cold lysis buffer containing 0.1 M Tris (pH 7.4), 150 mM NaCl, 10% sodium dodecyl sulfate (SDS), 1% Triton X, 0.5% Nonidet P-40 (NP40), 1 mM EDTA, 1 mM EGTA. Phosphatase inhibitor (2 μM sodium orthovanadate) and protease inhibitors (0.3 μM aprotinin, 21 μM leupetin, 1 μM pepstatin, 50 μM phenanthroline, 0.5 μM phenylmethylsulfonyl fluoride) were added to lysis buffer immediately prior to cell harvest. Cell lysates were sonicated and centrifuged (15,000*g,* 10 min, 4°C), and the supernatant transferred to a fresh tube. Protein content was determined by bicinchoninic acid (BCA) procedures according to manufacturer's (Pierce, Rockford, IL) instructions. Equal amounts of whole cell lysate protein were separated by SDS-PAGE electrophoresis. Cell lysates were mixed with loading buffer containing 0.25M Tris (pH6.8), 4% SDS, 10% glycerol, 0.01% bromophenol blue, and 10% dithiothreitol, then heated at 80°C for 5 min prior to electrophoresis. Proteins were resolved on SDS-polyacylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore corp., Billerica, MA). After transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4°C. Membranes were subsequently probed with horseradish peroxidase conjugated secondary antibodies for 2 hrs at room temperature. Membranes were immersed in ECL and data visualized by autoradiography using CL-XPosure film (Pierce).

RT-PCR. Total RNA was extracted and genomic DNA contamination was removed using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), according to manufacturer protocol. Total RNA was quantified with a Nanodrop ND-1000 spectrophotometer. Total RNA was reverse-transcribed to cDNA in a 10 μl reaction

volume using a high capacity cDNA reverse transcription kit (Applied Biosystems). The reverse transcription (RT) master mix containing RT buffer, deoxyribonucleotide triphosphate (dNTP) mix, RT random primers, RNase inhibitor (1.0 U/µl), and MultiScribe RT was added to 1 µg RNA and RNase-free water. Reverse transcription reaction conditions followed the protocol (25°C for 10 min, 37°C for 120 min, 85°C for 5 sec, followed by 4°C in definitely/ RT complete) and utilized the Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for cDNA synthesis. PCR amplification was run utilizing the 7500 fast system (Applied Biosystems) that consisted of enzyme activation at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec combined with annealing /extension at 60°C for 30 sec. All data were analyzed with the ABI 7500 real time PCR system. All TaqMan primer probes used in this study were also purchased from ABI. Data were recorded and analyzed with ABI Sequence Detector Software and graphs visualized with Excel software. All data were presented as mean ± standard error of the mean (SEM) and representative of duplicate determinations. Data were normalized to 18S and measured as relative differences using the $2^{-\Delta\Delta CT}$ method as previously described (35,36). Statistical analyses were conducted using SPSS. Differences in cell number were determined via student's *t*-test where a p-value of <0.05 was considered significant.

RNA Interference. Dicer-substrate short interfering RNA (siRNA) for HIF-1α specific sequences as well as non-targeting sequences were transfected using Lipofectamine RNAiMAX transfection reagent according to manufacturer's (Invitrogen) protocol. Briefly, 3T3-L1 preadipocytes were propagated in 6-well culture dishes until reaching confluence. Cells were then stimulated with 9 µl Lipofecatime RNAiMAX reagent and either 10 µM HIF-1α specific si RNA or non-targeting siRNA for 48 hr.

Growth medium was subsequently switched to differentiation medium containing MDI +/- OSM as described above.

Results

OSM Inhibits Proliferation. OSM was first discovered for its ability to inhibit cancer growth in the A375 human melanoma cell line (37). More recently, research has shown that OSM also inhibits osteosarcoma proliferation and reduces breast cancer in several cell lines (38,39). However, published reports also suggest that in some cell types, OSM induces proliferation and tumor growth (18,40). To investigate the effect of OSM on cell growth of proliferating PAs, subconfluent PAs were stimulated with OSM and cell count was measured each day by hemocytometer until untreated cells reached confluence (d6). As illustrated in Fig.3.2, OSM reduced PA proliferation relative to untreated controls. While statistical differences were reached by 2 days post-OSM treatment, by day 6, OSM-treated cells were ~50% less confluent that untreated. These data suggest that OSM inhibits subconfluent PA proliferation.

OSM Reduces Cyclins during Proliferation. To determine if OSM inhibits proliferation in PAs by mechanisms involving cell cycle, subconfluent PAs were stimulated with OSM and whole cell lysates were harvested over time. Lysates were immunoblotted for cyclin D1, cyclin A, cyclin B1 and p-RB. First, data reveal that OSM caused a considerable reduction in cyclin D1 protein by 24h compared to control. Additionally, although changing the media allowed for cell cycle progression, by 72h, cells treated with OSM had decreased levels of cyclin A, cyclin B1 and p-RB compared to control (Fig.3.3). As cyclin D1 promotes G1 phase progression and phosphorylation of RB to allow E2F-mediated S-phase gene transcription, these data suggest that OSM

causes cells to exit the cell cycle by a mechanism involving cyclin D1 suppression, which is not consistent with a G1/S phase block.

OSM Affects Cyclin D1 Protein and mRNA. Based on our finding that OSM greatly reduces cyclin D1 protein, we determined the effect of OSM on cyclin D1 protein and RNA. Subconfluent PAs were left untreated or treated with OSM and whole cell lysates and total RNA were collected over time. Cyclin D1 mRNA and protein expression was examined via RT-PCR and immunoblot analysis, respectively. As expected, media change accelerated cell cycle progression as indicated with increased levels of cyclin D1 protein and mRNA (Fig.3.4). Additionally, we confirmed our previous findings that OSM causes a greater reduction in cyclin D1 protein compared to control (Fig.3.4B). However, unlike previous reports by others suggesting that cyclin D1 is reduced by OSM through changes in protein stability (27), we found that OSM reduces cyclin D1 mRNA as early as 12h post-OSM treatment (Fig.3.4A). These data reveal that OSM causes PAs to drop out of the cell cycle very early by affecting levels of both cyclin D1 protein and mRNA.

OSM Inhibits Differentiation. Just as cell growth and proliferation of PAs are important factors in the development of obesity, the process of differentiation from PAs to mature ADs involves a cascade of events that cause changes in cell morphology, hormone sensitivity and gene expression that are important in the understanding of adipocyte cell function and how it relates to obesity (41). Importantly, the inhibition of differentiation can block adipose tissue expansion, which is recognized as causing insulin resistance (20). Previous research has shown that in some cell types, OSM inhibits differentiation (3,20,24–26). In order to confirm that OSM inhibits differentiation in our 3T3-L1 cell line, density-arrested PAs were stimulated with or without OSM in the presence of differentiation-inducer cocktail, MDI. Cells were harvested over a 6-day time

course and lysates were immunoblotted for differentiation markers C/EBPβ, PPARγ and ADPN, as well as OSMRβ. As illustrated in Fig.3.5, OSM reduced levels of all three differentiation markers. Of note, while OSMRβ was reduced over the course of differentiation in untreated cells, OSM caused an increased and sustained induction of OSMRβ in the presence of MDI. These data confirm the ability of OSM to inhibit adipocyte differentiation.

OSM Inhibits Differentiation during Clonal Expansion. The first phase of PA differentiation involves mitotic clonal expansion (MCE), which is characterized by cells synchronously progressing through at least one or more rounds of DNA replication or cell cycle during the first two days of differentiation (4). After MCE, cells then acquire the characteristics of mature adipocytes including alterations in cell shape and an increase in glucose transporters, insulin receptors and adipocyte proteins such as leptin and adipsin (4,41). To establish if MCE is a critical period for OSM's effect on differentiation, PAs were stimulated with or without OSM in the presence of MDI. OSM stimulation occurred on d0, d2, d4 or d6 and all cell lysates were collected on d8 and immunoblotted for differentiation markers PPARγ, aP2, C/EBPα and ADPN. As illustrated in Fig.3.6, addition of OSM on d0 and d2 prevented full induction of differentiation, whereas OSM beyond this time period had no effect of differentiation. These data suggest that OSM inhibits differentiation during clonal expansion.

OSM Reduces Cyclin D1 during Differentiation in a Dose-Dependent Manner. Our previous data suggest that the effect of OSM on proliferation is mediated through a reduction in cyclin D1. Because clonal expansion is characterized by postconfluent proliferation, we sought to determine if cyclin D1 plays a role in the effect of OSM on differentiation. Density-arrested PAs were stimulated with or without varying

concentrations of OSM in the presence of MDI and collected within MCE phase at 32 hr. Lysates were subjected to immunoblot analysis for cyclin D1. We observed a dose-dependent effect of OSM on cyclin D1 during MCE (Fig.3.7). These data suggest that cyclin D1 may be involved in the OSM inhibition of differentiation.

Effect of OSM on Differentiation Over Time. Our previous studies have shown that OSM induces HIF-1α. Research has shown that hypoxia inhibits differentiation and is dependent on HIF-1α (30,42–44). Additionally, in many cell types hypoxia causes cell cycle arrest (9,31,33,45,46). To explore the possibility that there is a link between the induction of HIF-1α and the reduction in cyclin D1 by OSM as it relates to the blockade of differentiation by OSM, we stimulated density-arrested PAs with or without OSM in the presence of MDI and collected lysates over the course of 6 days. Whole cell lysates were examined via immunoblot analysis for HIF-1α, HIF-1β, C/EBPβ, PPARγ, cyclin D1 and cyclin A. As expected, during the first 24 hrs, HIF-1α was strongly induced by OSM in the presence of MDI while HIF-1β remained constitutively expressed regardless of treatment. OSM also led to sustained induction of OSMRβ. OSM treatment in the presence of MDI did not inhibit the induction of C/EBPβ, but did accelerate its decay. OSM + MDI did however, completely inhibit the induction of C/EBPβ isoform, LAP1 and PPARγ. Additionally, while cyclin D1 was initially induced by MDI at 24 hr like HIF-1α, OSM caused it to be degraded rapidly (Fig.3.8). Unlike subconfluent PAs, OSM had no effect on cyclin A. These data indicate that the induction of HIF-1α by OSM correlates with the inhibition of cyclin D1 and differentiation.

Role for HIF-1α in the Effect of OSM on Differentiation. We observed that the induction of HIF-1α by OSM is concurrent with a reduction in cyclin D1 and the inhibition

of differentiation. To confirm a role for HIF-1α in the inhibition of differentiation, PAs were transfected with siRNA targeted to HIF-1α or non-targeting control sequences for 48 hr prior to stimulation with MDI with or without OSM. Whole cell lysates were harvested at 3 hr post-OSM, and at 2 days post-MDI + OSM. This experiment was repeated with whole cell lysates being collected at 3 hr post-OSM and then at 5 days post-MDI + OSM. HIF-1α protein was analyzed at 3 hr post-OSM for each separate experiment. Cyclin D1 and C/EBPβ protein was analyzed at 2 days post-MDI + OSM and terminal differentiation markers PPARγ, aP2 and ADPN were analyzed at 5 days post-MDI + OSM. Firstly, data indicate that transfection with siRNA targeted to HIF-1α greatly suppressed the accumulation of HIF-1α by OSM compared to cells transfected with non-targeting control sequences in both experiments (Fig.3.9A&C). Data demonstrate that knockdown of HIF-1α partially restored cyclin D1 but had no effect on C/EBPβ on d2 (Fig.3.9B). Moreover, suppression of HIF-1α partially restored C/EBPβ, including LAP1, PPARγ, aP2 and C/EBPα by OSM at d5 (Fig.3.9D). HIF-1α knockdown did not restore OSM's effect on ADPN. As C/EBPβ is required during MCE for PPARγ expression and our data show that it remains unaffected by OSM at d2, this suggests that HIF-1α may affect C/EBPβ activity. Collectively, these data indicate that the induction of HIF-1α by OSM plays a role in inhibiting differentiation.

Discussion

This report presents evidence demonstrating that cell growth and differentiation of adipocytes is regulated by the inflammatory cytokine OSM. First, we demonstrate that OSM directly inhibits the growth of proliferating PAs. Second, we show that OSM causes a reduction in cyclin D1, cyclin A, cyclin B1, and p-RB in PAs. Importantly, OSM caused

a great reduction to cyclin D1 mRNA and protein. Next we demonstrate that OSM inhibits differentiation during the clonal expansion phase and this correlates with an induction of HIF-1α and reduction of cyclin D1. Lastly, we show that HIF-1α plays a role in the OSM-mediated suppression of cyclin D1 during differentiation and regulators of adipocyte differentiation. Collectively, these data demonstrate a role for HIF-1α in the OSM-mediated inhibition of PA growth and differentiation.

Adipose tissue remodeling that is caused by obesity has been shown to be closely related to the function of adipose tissue (47). Adipocyte hyperplasia and hypertrophy contribute to adipose tissue expansion and cause downstream effects within the adipose tissue as a result of remodeling (5). For example, increases in inflammation and hypoxia that result from adipose tissue expansion cause a number of consequences, such as increased insulin resistance and adipocyte cell death (48). Although normal metabolic function relies on a delicate balance between pro-inflammatory and anti-inflammatory cytokines, obesity causes an increase in pro-inflammatory cytokine secretion, such as TNF-α, IL-6, IL-β and OSM (49). OSM was originally discovered for its ability to inhibit proliferation of a human melanoma cell line (37). However, further evidence has since demonstrated that the effect on proliferation is cell type dependent and in some cell types, OSM increases proliferation (27,40,50). Hyperplasia, or an increase in PA cell number, plays a major role in the development of adipose tissue (51,52). Therefore, to gain a better understanding of how PA proliferation is regulated during obesity, we addressed the hypothesis that OSM affects PA cell growth. Data presented here indicate that OSM dramatically reduces cellular proliferation. Others have shown that OSM inhibits proliferation by inhibiting cell cycle progression at the G1/S transition (27,53,54). Our data demonstrate that OSM

dramatically reduces cyclin D1, cyclin A and cyclin B1, as well as p-RB in proliferating PAs. The decline in cyclin D1 in our report indicates that these cells arrest early in G1 as opposed to late G1, which would be characterized by elevated cyclin D1, as reported by others (53,54). Due to the very pronounced effect of OSM on cyclin D1 and its regulatory role in G1 phase progression, we chose to focus on this cyclin in our following studies. Others have reported that OSM regulates cyclin D1 in skeletal muscle cells by ubiquitin/proteasomal-dependent degradation with no effect on cyclin D1 mRNA (27). While it is possible that cyclin D1 is also regulated by a post-transcriptional mechanism, data presented here demonstrate that OSM significantly reduces the expression of cyclin D1 mRNA indicating an inhibitory effect on transcription.

Adipogenesis, or the conversion of PAs to ADs has previously been targeted for antiobesity therapeutics. However, mounting evidence suggests that the blockade of adipocyte expansion is related to insulin resistance and type 2 diabetes (15). Other published reports have indicated that several cytokines such as TNF-α and IL-B, and also including OSM inhibit differentiation (24,25). However, the mechanism by which OSM inhibits differentiation of adipocytes is yet to be fully elucidated. Data presented in this dissertation here establish that OSM inhibits differentiation of adipocytes in the 3T3-L1 cell line. We demonstrate that OSM completely abrogates the induction of the "master regulators" of adipogenesis, PPARγ and C/EBPα. While OSM does not completely inhibit the induction of C/EBPβ, it accelerates its decay. Because the induction of C/EBPβ during MCE acts as a transcription factor to drive induction of PPARγ and C/EBPα (Fig.3.1B) (7,14), the fact that OSM inhibits PPARγ, but not C/EBPβ, raises the possibility that OSM may somehow inhibit the activation of C/EBPβ.

Along this premise, other reports have demonstrated that OSM does not affect mRNA or protein level of C/EBPβ, but does inhibit its activity (25).

We observed that the inhibitory effects of OSM on differentiation occurred only during the first days following hormonal induction kinetically coinciding with mitotic clonal expansion and C/EBPβ and preceding the expression of PPARγ and C/EBPα. Once the latter two transcriptional regulators were induced, cells became refractory to late stage stimulation. We explored the possibility that this refractory period was due to down regulation of the OSM receptor β (OSMRβ) as others have shown peak expression during the first 24 hrs followed by progressive decline in protein over the remaining course of differentiation (22). In refute of this hypothesis, however, we observed that continued stimulation with OSM throughout the course of differentiation prevented the progressive loss of receptor protein observed in the absence of OSM clearly demonstrating that the refractory period during late stages of differentiation was not due to down-regulation of the receptor. While the mechanism promoting OSMRβ accumulation in the presence of OSM stimulation in adipocytes has yet to be determined, these data are consistent with other reports showing that the expression of OSMRβ is up-regulated by OSM stimulation in fetal hepatocytes and granulosa cells (55,56). Collectively, these data indicate that OSM impinges on differentiation only during early phases characterized by C/EBPβ and MCE.

The early phase of adipocyte differentiation is characterized by several rounds of cell cycle during MCE, as well as the induction of a cascade of transcription factors that drive terminal differentiation (Fig.3.1) (7,41). Our previous data demonstrated a significant effect of OSM on cyclin D1. Cyclin D1 is critical for progression through G1 phase during MCE by inactivating RB to allow passage through the restriction point for

DNA synthesis (57). Evidence also suggests that cyclin D1 directly interacts with C/EBPβ during early differentiation in a cyclin dependent kinase manner (13,57). Data presented in our report demonstrate that OSM reduces cyclin D1 in a dose-dependent manner during MCE. This raises that possibility that the loss of cyclin D1 may be involved in the inhibition of differentiation. Further analyses exploring the effect of OSM over six consecutive days of differentiation revealed that OSM stimulation suppressed cyclin D1 by day 2 of differentiation, with little affect on cyclin A until day 4. These data suggest that suppression of cyclin D1 temporality occurred after cells had moved beyond the restriction point leading to cyclin A expression and S phase progression. Our data indicate that OSM causes an early decay of cyclin D1 and inhibits the induction of the "master regulator" of adipogenesis, PPARγ. It is widely accepted that MCE, which includes the early induction of cyclin D1, is required for terminal differentiation (7). This suggests that there is a critical level and timing of cyclin D1 induction needed for differentiation.

Our unpublished data and others (28) demonstrate that OSM can regulate HIF-1α in normoxic conditions. Consistently, data presented here indicate that OSM induces HIF-1α during early phases of differentiation concurrently with the induction of cyclin D1 and C/EBPβ. This raises the possibility that OSM may regulate cyclin D1 through HIF-1α and affect differentiation through a cell cycle independent mechanism. It is well established that for HIF-1α to act as a functional transcription factor, it must heterodimerize with HIF-1β (58,59). Our data suggest that HIF-1α is functional during MCE upon the stimulation with OSM due to the constitutively expressed HIF-1β. A published report found that OSMRβ-deficient mice had increased insulin resistance (60). Our data demonstrated OSM led to sustained OSMRβ in the presence of MDI and to the

inhibition of differentiation. Given that the blockade of adipogenesis increases insulin resistance, our data would suggest that OSMRβ does not protect against insulin resistance.

Previous research indicates that hypoxia inhibits adipocyte differentiation and that HIF-1α is involved in this inhibition (30,43,61). As our data has shown that OSM induces HIF-1α and that OSM inhibits differentiation, it is conceivable that HIF-1α plays a role in the OSM-mediated blockade of adipogenesis. Data in this investigation demonstrate that the effect of OSM in late stages (d5) of differentiation on C/EBPβ, PPARγ, aP2 and C/EBPα is rescued by HIF-1α knockdown. However, HIF-1α knockdown did not rescue the effect of OSM on ADPN inhibition. These data confirm a partial role for HIF-1α in OSM-mediated inhibition of differentiation. As OSM is a pleiotropic cytokine affecting a variety of genes and processes and we would not expect the inhibition of differentiation by OSM to be solely regulated through HIF-1α. Moreover, examination of the effect of HIF-1α knockdown in the presence of OSM during MCE on d2 of differentiation suggests that HIF-1α is involved in the regulation of cyclin D1 by OSM. OSM, regardless of HIF-1α, had no effect on the induction of C/EBPβ during MCE. This is consistent with literature that suggests hypoxia has minimal effect on C/EBPβ (30). However, this finding is surprising since C/EBPβ binds directly to the promoter regions of C/EBPα and PPARγ to induce their expression (17). This is consistent, however, with other research indicating that OSM does not affect the expression of C/EBPβ (25). This raises the possibility that C/EBPβ in the presence of OSM may not be functional. Several studies have proposed that C/EBPβ is auto-inhibited and must be derepressed for functionality (62,63). As mentioned earlier, some

research demonstrates the interaction between cyclin D1 and C/EBPβ for terminal differentiation (13). This raises the possibility that there was enough cyclin D1 present in early MCE to activate cyclin A for cell cycle progression but not enough to activate C/EBPβ for terminal differentiation. Collectively, our data demonstrate an essential role for HIF-1α in the OSM-mediated inhibition of differentiation, although the mechanisms by which this occurs remains unresolved.

In summary, data presented in this report demonstrate that OSM affects adipose tissue remodeling by impacting cellular proliferation and adipocyte differentiation. Our data provide evidence that OSM inhibits adipogenesis specifically during early phases of differentiation, which includes MCE. Our data also indicate that OSM significantly reduces cyclin D1 in both subconfluent proliferating PAs and during adipogenesis. While the reduction of cyclin D1 in proliferating PAs likely affected cell growth by a mechanism related to cell cycle, our data suggest that the effect of OSM on cyclin D1 during MCE likely inhibited differentiation by a cell cycle independent mechanism. We also report that the loss of HIF-1α partially restored cyclin D1 and differentiation by restoration of adipogenic factors such as PPARγ and C/EBPα. Although further studies are needed to elucidate the regulatory mechanism by which OSM-mediated HIF-1α induction inhibits differentiation, this study establishes a platform for future investigations, especially in regards to the relationship between cyclin D1 and C/EBPβ. As studies continue to examine the regulation of cell growth and differentiation by OSM and HIF-1α, insights into these mechanisms will provide greater understanding as to how inflammation and hypoxia lead to adipose tissue remodeling.

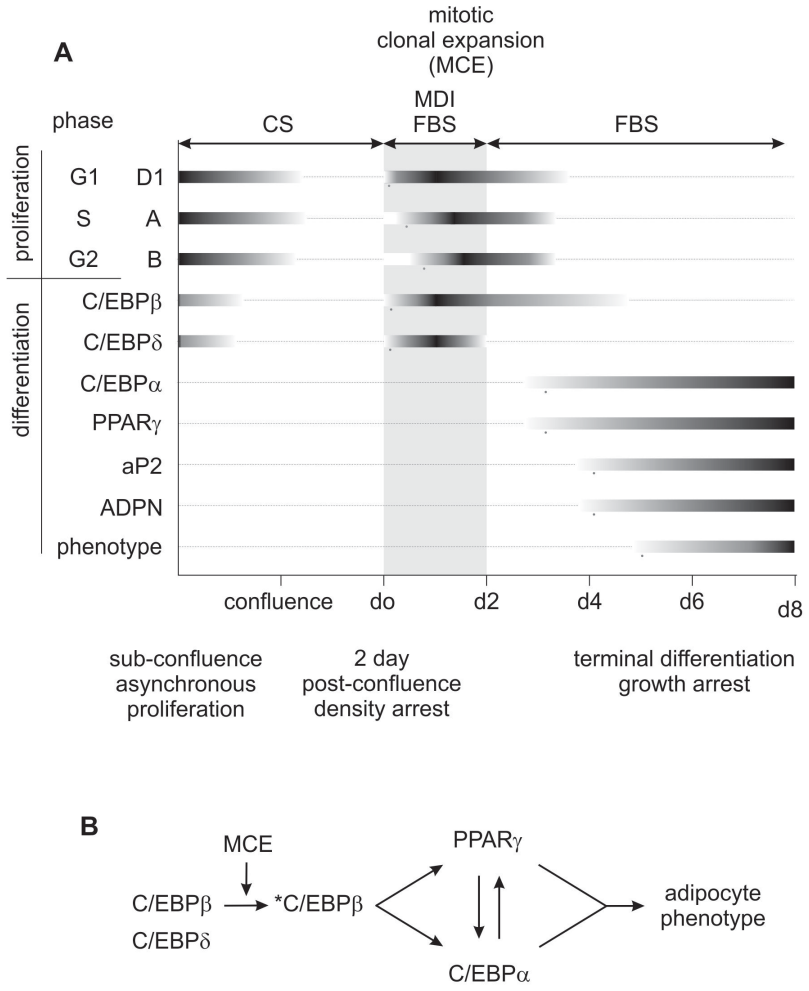


Figure 3.1. Characterization of Regulatory Factors during Adipose Tissue Hyperplasia. A) During subconfluent asynchronous proliferation, cells are constantly undergoing cell cycle and express varying levels cyclin D1, cyclin A, cyclin B, C/EBPβ and C/EBPδ. Upon stimulation with MDI at 2d post-confluence, cells undergo synchronous cell cycle during MCE, in which cyclin D1 is induced first and drives subsequent increases in cyclin A and cyclin B. MCE is also characterized by increases in C/EBPβ and C/EBPδ, which are obligatory for downstream targets such as C/EBPα and PPARγ, which lead to the phenotype of mature adipocytes. B) During MCE, C/EBPβ's auto-inhibition is repressed, allowing for the induction of PPARγ and C/EBPα, which cross-regulate each other to maintain differentiation and drive the adipocyte phenotype.

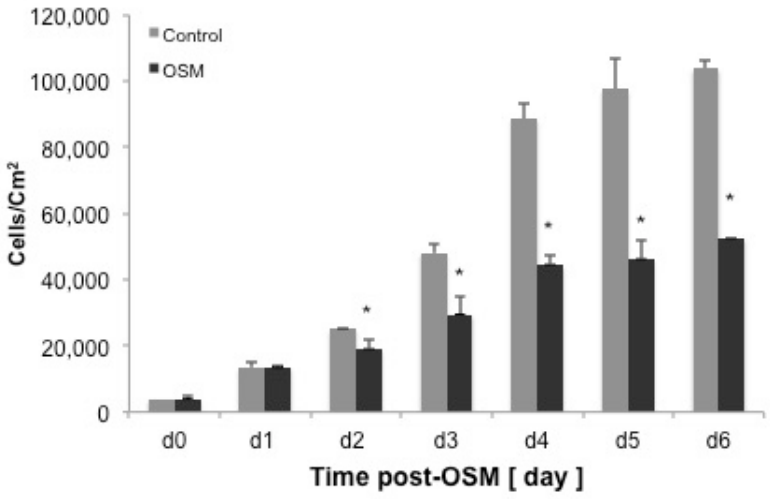


Figure 3.2. OSM Inhibits Proliferation. Proliferating preadipocytes were stimulated with 1nM OSM. Cell count was measured every day until control cells reached confluence (d6). Statistical significance was determined by Student's *t*-test (*p<0.05).

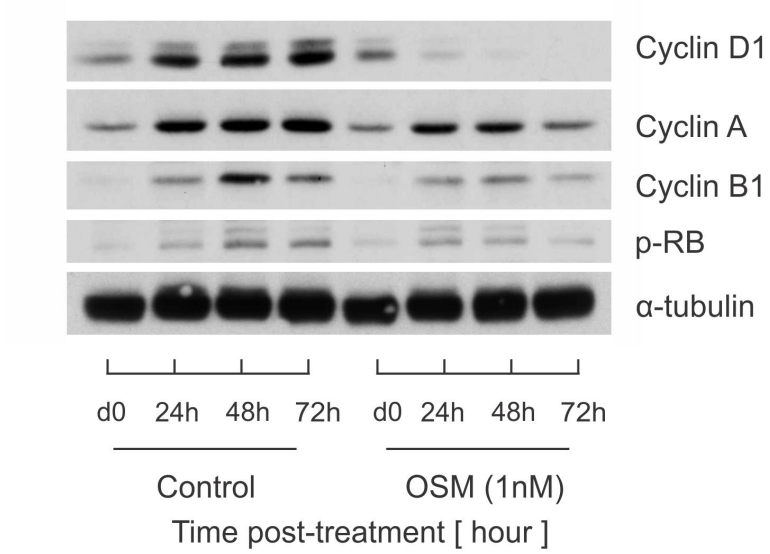
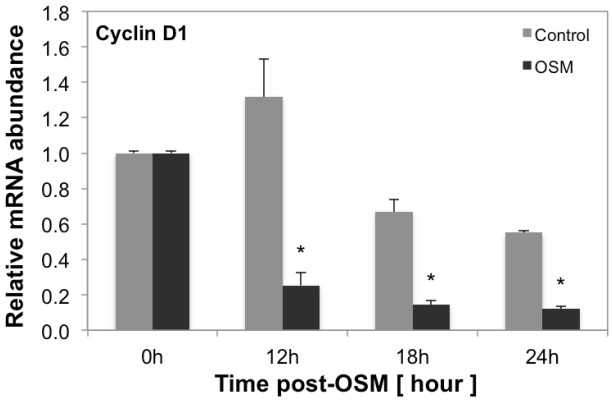


Figure 3.3. OSM Reduces Cyclins during Proliferation. Proliferating preadipocytes were stimulated with 1nM OSM and cell lysates were harvested over time. Protein expression of cyclin D1, cyclin A, cyclin B1, p-RB and α-tubulin was analyzed via immunoblotting.

A



B

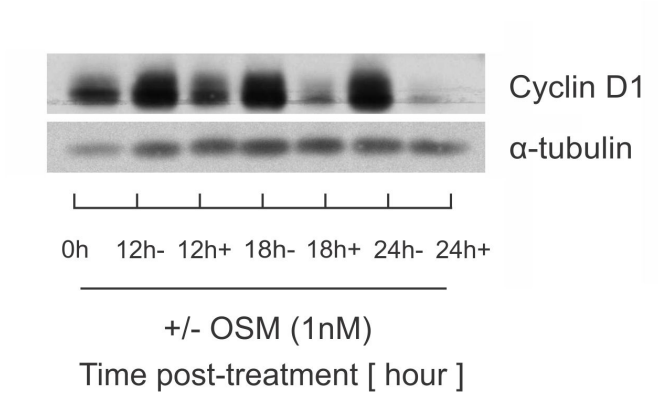


Figure 3.4 OSM Affects Cyclin D1 Protein and mRNA. Proliferating preadipocytes were stimulated with 1nM OSM. A) Total RNA was collected and gene expression of cyclin D1 was measured via qRT-PCR. All data were normalized to 18S rRNA and expressed relative to untreated cells (0h). Statistical significance was determined by Student's *t*-test (*$p<0.05$). B) Cell lysates were collected over time and protein expression of cyclin D1 and α-tubulin was analyzed via immunoblotting.

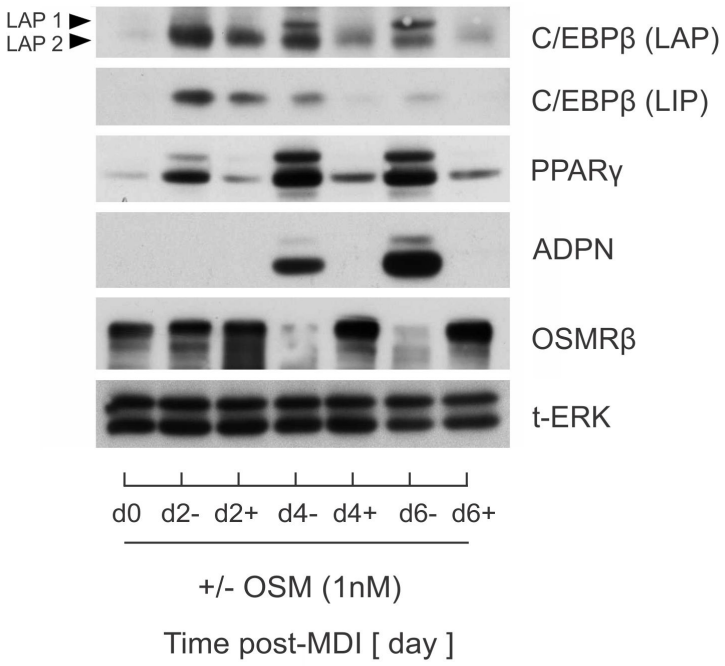


Figure 3.5 OSM Inhibits Differentiation. Preadipocytes were stimulated to differentiate with the hormonal cocktail MDI as outlined above and with 1 nM OSM. Cell lysates were collected over a 6-day time course and protein expression of C/EBPβ, PPARγ, ADPN, OSMRβ and total-ERK was measured via immunoblot analysis.

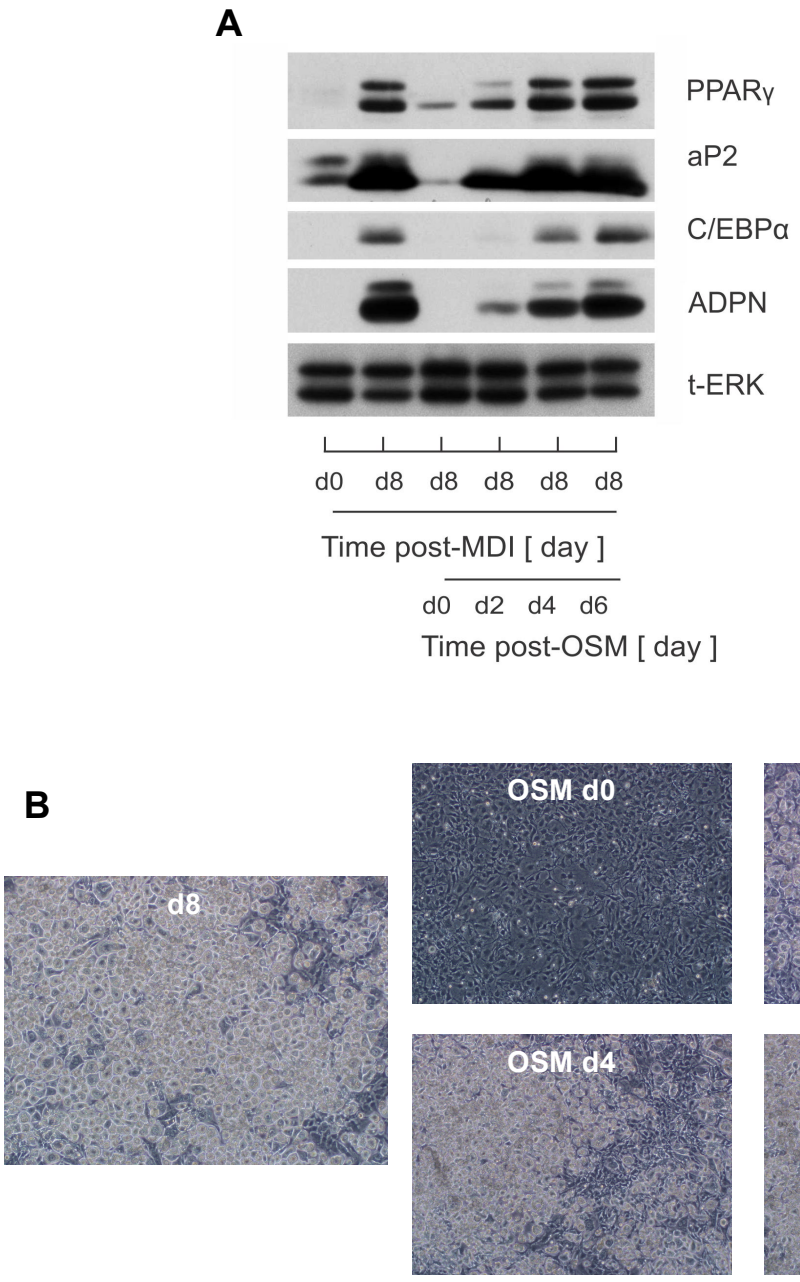


Figure 3.6. OSM Inhibits Differentiation during Clonal Expansion. Preadipocytes were stimulated to differentiation with MDI and stimulation of 1 nM OSM occurred every 2 days for 6 days. A) Cell lysates were all collected on d8 prior to immunoblot analysis of PPARγ, aP2, C/EBPα, ADPN and total-ERK. B) Examination of cell morphology of untreated preadipocytes or treated with OSM. Cells were assessed for lipid accumulation using phase contrast microscopy.

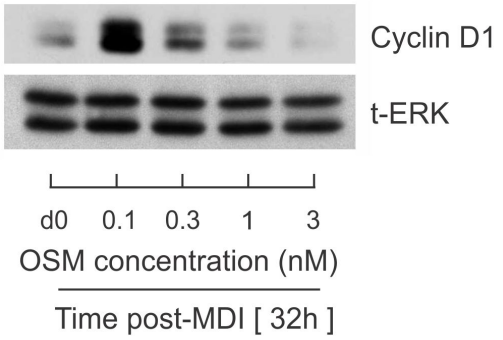


Figure 3.7. OSM Reduces Cyclin D1 during Differentiation in a Dose-Dependent Manner. Preadipocytes were stimulated to differentiate with MDI concurrent with varying concentrations of OSM stimulation. Cell lysates were collected at 32h post-MDI and analyzed for protein expression of cyclin D1 and total-ERK by immunoblotting.

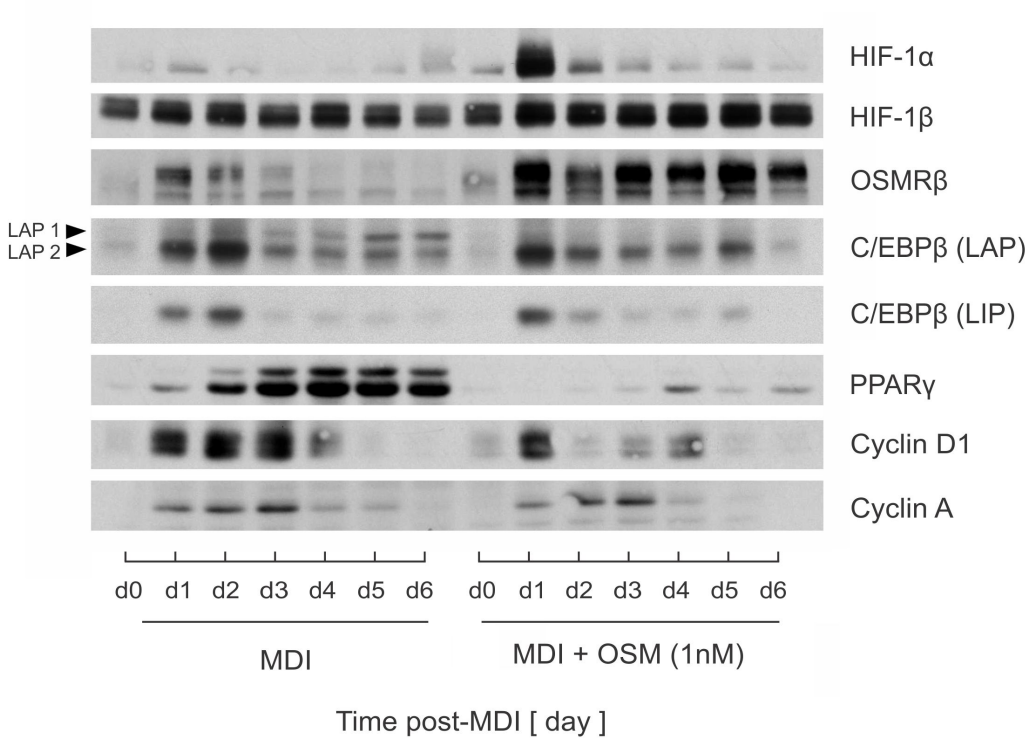


Figure 3.8. Effect of OSM on Differentiation Over Time. Preadipocytes were stimulated to differentiate with MDI in the presence or absence of 1nM OSM. Cell lysates were collected over a 6-day time course prior to immunoblot analysis for HIF-1α, HIF-1β, OSMRβ, PPARγ, cyclin D1 and cyclin A.

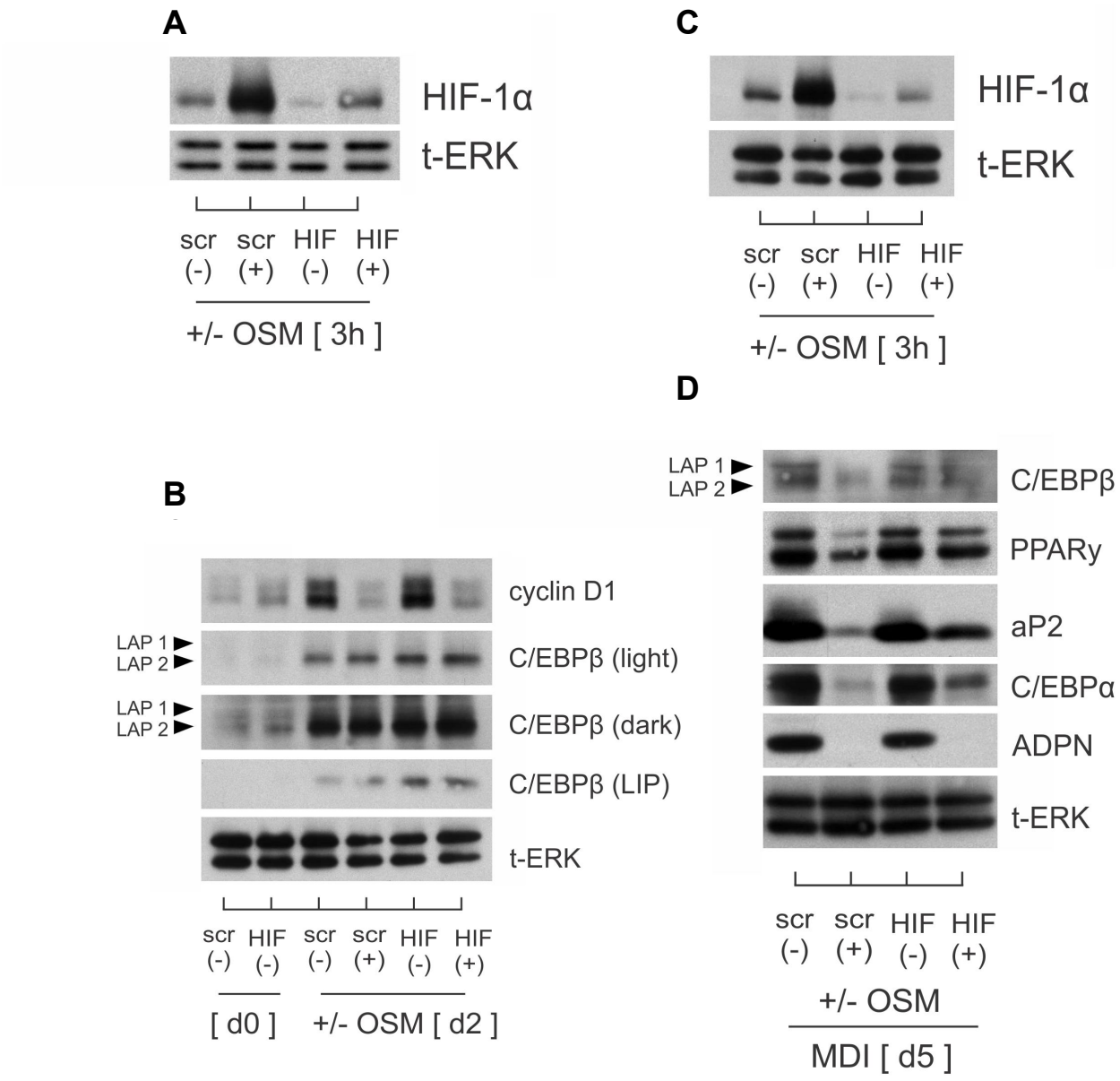


Figure 3.9. Role for HIF-1α in the Effect of OSM on Differentiation. Preadipocytes were transfected with Lipofectamine RNAiMAX reagent in the presence of non-targeting control siRNA or siRNA for HIF-1α for 48h prior to stimulation with MDI or MDI + OSM. A) Cell cell lysates were collected at 3h post-OSM prior to immunoblot analysis for HIF-1α and total-ERK. B) Cell lysates were collected at 2d post-MDI +/- OSM prior to immunoblot analysis for cyclin D1, C/EBPβ and total-ERK. C) Cell lysates were collected at 3h post-OSM prior to immunoblot analysis for HIF-1α and total-ERK. D) Cell lysates were collected at 5d post-MDI +/- OSM prior to immunoblot analysis for C/EBPβ, PPARγ, aP2, C/EBPα, ADPN and total-ERK.

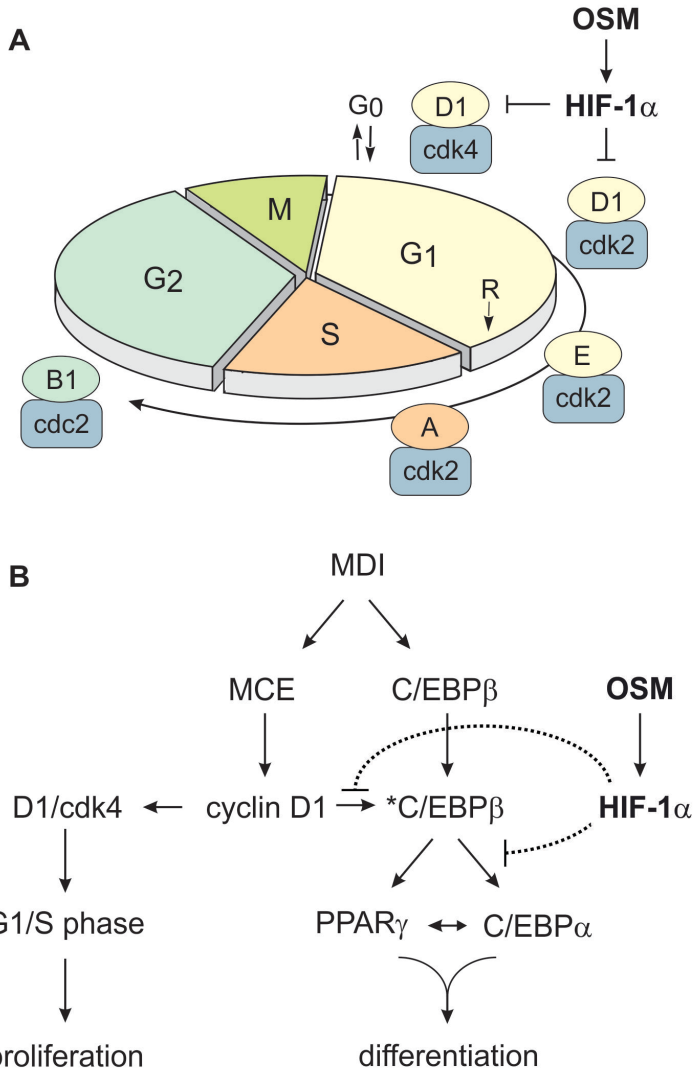


Figure 3.10. Proposed Working Model for Role of HIF-1α in OSM-Mediated Effects on Preadipocyte Proliferation and Differentiation. A) OSM increases HIF-1α, which causes a reduction in cyclin D1 to inhibit subconfluent proliferation in preadipocytes. B) Differentiation cocktail MDI normally induces synchronous proliferation during MCE where cyclin D1 drives cell cycle by cdk4-dependent mechanism (left). When OSM is present during MDI stimulation, the induction of HIF-1α causes cyclin D1 to decay and inhibit the functional activity of C/EBPβ, which is critical for the induction of PPARγ and C/EBPα.

Reference List

1. Trayhurn P. Endocrine and signalling role of adipose tissue: New perspectives on fat. Acta Physiol Scand. 2005;184(4):285–93.

2. Chen XH, Zhao YP, Xue M, Ji CB, Gao CL, Zhu JG, et al. TNF-α induces mitochondrial dysfunction in 3T3-L1 adipocytes. Mol Cell Endocrinol. 2010;328(1–2):63–9.

3. White UA, Stewart WC, Stephens JM. Gp130 cytokines exert differential patterns of crosstalk in adipocytes both in vitro and in vivo. Obesity (Silver Spring). 2011;19(5):903–10.

4. Moreno-Navarrete JM, Fernández-real JM. Adipocyte Differentiation. Adipose Tissue Biology. 2012. 17-39 p.

5. Sun K, Kusminski CCM, Scherer PEP. Adipose tissue remodeling and obesity. J Clin Invest. 2011;121(6):2094–101.

6. Fernandes KM, Auld CA, Hopkins RG, Morrison RF. Helenalin-mediated post-transcriptional regulation of p21(Cip1) inhibits 3T3-L1 preadipocyte proliferation. J Cell Biochem. 2008;105(3):913–21.

7. Tang Q-Q, Otto TC, Lane MD. Mitotic clonal expansion: A synchronous process required for adipogenesis. Proc Natl Acad Sci. 2003;100(1):44–9.

8. Auld CA, Fernandez KM MR. Skp2-Mediated p27(Kip1) Degradation During S/G2 Phase Progression of Adipocyte Hyperplasia. J Cell Physiol. 2007;211:101–11.

9. Ortmann B, Druker J, Rocha S. Cell cycle progression in response to oxygen levels. Cell Mol Life Sci. 2014;71(18):3569–82.

10. Orford KW SD. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nat Rev Genet. 2008;115–28.

11. Bertoli, C, Skotheim JM de BR. Control of cell cycle transition during G1 and S phase. Nat Rev Mol Cell Biol. 2013;14(8):518–28.

12. Fu M, Rao M, Bouras T, Wang C, Wu K, Zhang X, et al. Cyclin D1 Inhibits Peroxisome Proliferator-activated Receptor gamma-mediated Adipogenesis through Histone Deacetylase Recruitment *. 2005;280(17):16934–41.

13. Liu Q, Boudot A, Ni J, Hennessey T, Beauparlant SL, Rajabi HN, et al. Cyclin D1 and C/EBPβ LAP1 Operate in a Common Pathway To Promote Mammary Epithelial Cell Differentiation. Mol Cell Biol. 2014;34(16):3168–79.

14. Tang Q, Zhang J, Lane MD. Sequential gene promoter interactions of C/EBP b , C/EBP a , and PPAR c during adipogenesis. Biochem Biophys Res Commun. 2004;319:235–9.

15. Sarjeant K, Stephens JM. Adipogenesis. Cold Spring Harb Perspect Biol. 2012;4(9):1–19.

16. Tang QQ, Lane MD. Adipogenesis : From Stem Cell to Adipocyte. Annu Rev Biochem. 2012;(81):715–36.

17. Guo L, Li X, Tang QQ. Transcriptional regulation of adipocyte differentiation: A central role for CCAAT/ enhancer-binding protein (C/EBP) β. J Biol Chem. 2015;290(2):755–61.

18. Richards CD. The Enigmatic Cytokine Oncostatin M and Roles in Disease. ISRN Inflamm. 2013;512103.

19. White UA, Stephens JM. The gp130 Receptor Cytokine Family : Regulators of Adipocyte Development and Function. Curr Pharm Des. 2011;17(4):340–6.

20. Elks CM, Stephens JM. Oncostatin m modulation of lipid storage. Biology (Basel). 2015;4(1):151–60.

21. Hermanns HM. Oncostatin M and interleukin-31: Cytokines, receptors, signal transduction and physiology. Cytokine Growth Factor Rev. 2015;26(5):545–58.

22. Sanchez-Infantes D, White UA, Elks CM, Morrison RF, Gimble JM, Considine R V., et al. Oncostatin M is produced in adipose tissue and is regulated in conditions of obesity and type 2 diabetes. J Clin Endocrinol Metab. 2014;99(2):217–25.

23. Elks CM, Zha P, Grant RW, Hang H, Bailey JL, Burk DH, et al. Loss of Oncostatin M Signaling in Adipocytes Induces Insulin Resistance and Adipose Tissue Inflammation in Vivo*. J Biol Chem. 2016;291(33):17066–76.

24. White UA, Stewart WC, Mynatt RL, Stephens JM. Neuropoietin attenuates adipogenesis and induces insulin resistance in adipocytes. J Biol Chem. 2008;283(33):22505–12.

25. Miyaoka Y, Tanaka M, Naiki T, Miyajima A. Oncostatin M inhibits adipogenesis through the RAS/ERK and STAT5 signaling pathways. J Biol Chem. 2006;281(49):37913–20.

26. Song HY, Jeon ES, Kim J Il, Jung JS, Kim JH. Oncostatin M promotes osteogenesis and suppresses adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells. J Cell Biochem. 2007;101(5):1238–51.

27. Kim H, Jo C, Jang BG, Oh U, Jo SA. Oncostatin M induces growth arrest of skeletal muscle cells in G1 phase by regulating cyclin D1 protein level. Cell Signal. 2008;20(1):120–9.

28. Vollmer S, Kappler V, Kaczor J, Flügel D, Rolvering C, Kato N, et al. Hypoxia-inducible factor 1α is up-regulated by oncostatin M and participates in oncostatin M signaling. Hepatology. 2009;50(1):253–60.

29. Trayhurn P. Hypoxia and Adipocyte Physiology: Implications for Adipose Tissue Dysfunction in Obesity. Annu Rev Nutr. 2014;34(1):207–36.

30. Lin Q, Lee YJ, Yun Z. Differentiation arrest by hypoxia. J Biol Chem. 2006;281(41):30678–83.

31. Krtolica a, Krucher N a, Ludlow JW. Hypoxia-induced pRB hypophosphorylation results from downregulation of CDK and upregulation of PP1 activities. Oncogene. 1998;17(18):2295–304.

32. Goda Nobuhito, Dozier Sara J, Johnson RS. HIF-1 in Cell Cycle Regulation, Apoptosis and Tumor Progression. Anal Chem. 2005;7(55):804–13.

33. Goda N, Ryan HE, Khadivi B, McNulty W, Rickert RC, Johnson RS. Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. Mol Cell Biol. 2003;23(1):359–69.

34. Yun Z, Maecker HL, Johnson RS, Giaccia AJ. Inhibition of PPAR-gamma2 Gene Expression by the HIF-1-Regulated Gene DEC1/Stra13 : A Mechanism for Regulation of Adipogenesis by Hypoxia. Dev Cell. 2002;2:331–41.

35. Ferguson BS, Nam H, Hopkins RG, Morrison RF. Impact of reference gene selection for target gene normalization on experimental outcome using real- time qRT-PCR in adipocytes. PLoS One. 2010;5(12).

36. Livak Kenneth J STD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T))Method. Methods. 2001;25(4):402–8.

37. Zarling JM, Shoyab M, Marquardt H, Hanson MB, Lioubin MN, Todaro GJ. Oncostatin M : A growth regulator produced by differentiated histiocytic lymphoma cells. Proc Natl Acad Sci. 1986;83(December):9739–43.

38. Berreur M, Pradal G, Colombeix C, Heymann D. Downregulation of Osteoblast Markers and Induction of the Glial Fibrillary Acidic Protein by Oncostatin M in Osteosarcoma Cells Require PKC delta and STAT3. J Bone Miner Res. 2004;19:1850–61.

39. Douglas AM, Goss GA, Sutherland RL, Hilton DJ, Berndt MC, Nicola NA, et al. Expression and function of members of the cytokine receptor superfamily on breast cancer cells. Oncogene. 2010;14(1997):661–9.

40. Zou F, Xu JC, Wu GH, Zhou LL, Wa QD, Peng JQ, et al. Effects of oncostatin M on cell proliferation and osteogenic differentiation in C3H10T1/2. J Musculoskelet Neuronal Interact. 2016;16(4):377–85.

41. Regina P Brun, Jae B Kim, Erding Hu SA and BMS. Adipocyte differentiation : a transcriptional regulatory cascade. Curr Opin Cell Biol. 1996;8:826–32.

42. Trayhurn P. Hypoxia and Adipose Tissue Function and Dysfunction in Obesity. Physiol Rev. 2013;93:1–21.

43. Wang B, Wood IS, Trayhurn P. Hypoxia induces leptin gene expression and secretion in human preadipocytes: Differential effects of hypoxia on adipokine expression by preadipocytes. J Endocrinol. 2008;198(1):127–34.

44. Carrière A, Carmona MC, Fernandez Y, Rigoulet M, Wenger RH, Pénicaud L, et al. Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: A mechanism for hypoxia-dependent effect. J Biol Chem. 2004;279(39):40462–9.

45. Hubbi ME, Gilkes DM, Hu H, Kshitiz, Ahmed I, Semenza GL. Cyclin-dependent kinases regulate lysosomal degradation of hypoxia-inducible factor 1α to promote cell-cycle progression. Proc Natl Acad Sci USA. 2014;111(32):E3325-34.

46. Bae S, Jeong HJ, Cha HJ, Kim K, Choi YM, An IS, et al. The hypoxia-mimetic agent cobalt chloride induces cell cycle arrest and alters gene expression in U266 multiple myeloma cells. Int J Mol Med. 2012;30(5):1180–6.

47. Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB. Adipose tissue remodeling: Its role in energy metabolism and metabolic disorders. Front Endocrinol (Lausanne). 2016;7(APR):1–16.

48. Wood IS, De Heredia FP, Wang B, Trayhurn P. Cellular hypoxia and adipose tissue dysfunction in obesity. Proc Nutr Soc. 2009;68(4):370–7.

49. Esser N, Legrand-Poels S, Piette J, Scheen AJ, Paquot N. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. Diabetes Res Clin Pract. 2014;105(2):141–50.

50. Hutt JA, Dewille JW. Oncostatin M Induces Growth Arrest of Mammary Epithelium via a CCAAT/enhancer-binding Protein delta -dependent Pathway 1. Mol Cancer Ther. 2002;1(June):601–10.

51. Wagner G, Lindroos-christensen J, Einwallner E, Husa J, Zapf T, Lipp K, et al. HO-1 inhibits preadipocyte proliferation and differentiation at the onset of obesity via ROS dependent activation of Akt2. Nat Publ Gr. 2017;(December 2016):1–17.

52. Fernandez Y, Rigoulet M, Pe L. Inhibition of preadipocyte proliferation by mitochondrial reactive oxygen species. Fed Eur Biochem Soc. 2003;550:163–7.

53. Klausen P, Pedersen L, Jurlander J, Baumann H. Oncostatin M and interleukin 6 inhibit cell cycle progression by prevention of p27kip1 degradation in HepG2 cells. Oncogene. 2000;19(32):3675–83.

54. Halfter H, Friedrich M, Resch A, Kullmann M, Stögbauer F, Ringelstein EB, et al. Oncostatin M induces growth arrest by inhibition of Skp2, Cks1, and cyclin A expression and induced p21 expression. Cancer Res. 2006;66(13):6530–9.

55. Goetten ALF, Ferst JG, Lucia T, Duggavathi R, Portela VVM, Bordignon V, et al. Oncostatin M and its receptors mRNA regulation in bovine granulosa and luteal cells. Theriogenology. 2018;125:324–30.

56. Kamiya A. Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. EMBO J. 2002;18(8):2127–36.

57. Fu M, Wang C, Li Z, Sakamaki T, Pestell RG. Minireview: Cyclin D1: Normal and abnormal functions. Endocrinology. 2004;145(12):5439–47.

58. Weidemann A, Johnson RS. Biology of HIF-1α. Cell Death Differ. 2008;15(4):621–7.

59. Dengler VL, Galbraith MD, Espinosa JM. Transcriptional regulation by hypoxia inducible factors. Crit Rev Biochem Mol Biol. 2014;49(1):1–15.

60. Komori T, Tanaka M, Senba E, Miyajima A, Morikawa Y. Lack of oncostatin M receptor beta leads to adipose tissue inflammation and insulin resistance by switching macrophage phenotype. J Biol Chem. 2013;288(30):21861–75.

61. Sahai A, Patel MS, Zavosh AS, Tannen RL. Chronic hypoxia impairs the differentiation of 3T3-L1 fibroblast in culture: Role of sustained protein kinase C activation. J Cell Physiol. 1994;160(1):107–12.

62. Lee S, Miller M, Shuman JD, Johnson PF. CCAAT/enhancer-binding protein β DNA binding is auto-inhibited by multiple elements that also mediate association with p300/CREB-binding protein (CBP). J Biol Chem. 2010;285(28):21399–410.

63. Kowenz-Leutz E, Twamley G, Ansieau S, Leutz A. Novel mechanism of C/EBPβ (NF-M) transcriptional control: Activation through derepression. Genes Dev. 1994;8(22):2781–91.

CHAPTER IV

EPILOGUE

Data presented in this dissertation represent evidence demonstrating the regulation of hypoxia inducible factor-1α (HIF-1α) by Oncostatin M (OSM) during normoxia and specific downstream adaptations in 3T3-L1 adipocytes. Findings presented in Ch.2 collectively demonstrated that OSM, through several well-known signaling pathways, transcriptionally regulates HIF-1α and leads to metabolic adaptations during normoxic conditions. Data from Ch.3 demonstrated that (OSM) regulates adipose tissue remodeling by reducing cellular growth and differentiation and that these effects are, in part, mediated by HIF-1α. Collectively, data presented in this dissertation provide evidence for the regulation of HIF-1α by OSM during normoxia and the overall adaptations made by adipocytes in response to OSM-mediated HIF-1α induction, linking hypoxia and inflammation during obesity. While data from this dissertation established a role for HIF-1α in the effect of OSM on adipocytes, questions for future investigation have been generated. This chapter will discuss findings from previous chapters and focus on potential areas of research.

Data presented in Ch.2 demonstrated that inflammatory cytokine OSM induces HIF-1α in preadipocytes (PAs) under normoxic conditions. We also found that TNF-α induced HIF-1α (data not shown), but the induction was weak in comparison. Our preliminary findings demonstrated that HIF-1α induction by OSM was as great as the induction by $CoCl_2$, a well-known hypoxia mimetic. However, it differed in its kinetics and

mechanisms, with OSM-mediated regulation being dependent on transcription. However, examining which sequences and regions of the HIF-1α promoter are required for OSM to induce HIF-1α transcription would give a more detailed insight into regulatory mechanisms. Preliminary findings from our lab and others have demonstrated that OSM activates a variety of signaling pathways that link OSM to its downstream effects (1–4). While this dissertation was limited to signaling pathway inhibitors to examine their role in OSM-mediated HIF-1α induction, studies involving knockdown of signaling pathways to examine mRNA and protein induction is critical for understanding which pathways link OSM to HIF-1α transcription. Although our preliminary studies examined only STAT3, ERK and AKT signaling pathways, other pathways have been shown to respond to OSM, such as STAT5 (4).

In order to act as a transcription factor, it is a requirement for HIF-1α to translocate to the nucleus and heterodimerize with HIF-1β (5,6). HIF-1α can then bind the core consensus sequence within the hypoxia responsive element (HRE) of HIF-1α target genes (5). While our studies did find that OSM-induced HIF-1α was bound to HIF-1β for functionality, our research did not investigate which signaling pathways activated by OSM increased HRE activity. An interesting addition to the story would be to knockdown several of the signaling pathways in the presence of OSM and examine the HRE promoter activity. These data would further confirm the role of critical signaling pathways that link OSM to the downstream HIF-1α mediated effects.

It is well known that hypoxia regulates many different genes encoding for a variety of biological processes, such as cellular proliferation, angiogenesis, inflammation and glycolysis (7,8). Our data confirmed that OSM regulates genes involved in the same processes as hypoxia and did so through mechanisms involving HIF-1α. Our data

demonstrated that glycolytic genes as well as genes associated with angiogenesis were up-regulated by OSM in a HIF-1α-dependent manner. However, investigations examining the effect of OSM on the promoter regions of these genes, as well as gene promoter constructs mutated at the HRE, would increase our understanding of precise mechanisms by which OSM regulates these genes. We also found a role for HIF-1α in OSM-mediated increases in glucose uptake and lactate production, which supports an increase in glycolytic function. Seahorse biotechnology allows researchers to measure oxygen consumption rate and extracellular acidification rate of cells in real time. Although we attempted to measure glycolytic function of adipocytes in response to OSM, using the Seahorse, the variability of this technology made it difficult to determine the true bioenergetics. However, future investigations to optimize conditions for these cells and determine a role for both OSM and HIF-1α in adipocyte bioenergetics would likely support a shift in metabolism toward glycolytic flux.

Our data further support a role for HIF-1α in OSM-mediated metabolic adaptation through the observation that the angiogenic factors, VEGF and PAI-1 were increased. However, exploring the secretion of VEGF and PAI-1 by adipocytes in response to OSM and HIF-1α would further confirm a role for vascularization. Published reports have demonstrated that hypoxia increases the production of a conglomeration of adipokines such as leptin, fasting induced adipose factor (FIAF), interleukin-6 (IL-6), macrophage migration inhibitory factor (MIF) and others (9–13). Other reports show evidence of the effect of OSM on adipokine induction such as tissue inhibitor metalloproteinases 1 (TIMP1) and insulin-like growth factor-binding protein 3 (IGFBP3) (14). Future investigations of the role of HIF-1α in the effect of OSM on adipokines known to be

associated with hypoxia, including those unrelated to angiogenesis, would provide additional evidence for the link between inflammation and hypoxia found during obesity.

Observations presented above highlight our preliminary data and future studies involving the regulation of HIF-1α by OSM and the role of this relationship in metabolic adaptation, such as glycolysis and vascularization. In this dissertation we have also identified a role for HIF-1α in the regulation of adipose tissue remodeling by OSM (Ch.3). While our preliminary studies revealed that OSM inhibited subconfluent PA proliferation, we mostly focused on the reduction of cyclin D1 as the mechanism of inhibition of cell growth. However, studies analyzing cell cycle with flow cytometry would contribute greatly to our understanding of how OSM regulates cell cycle progression in proliferating PAs. Additionally, our preliminary data demonstrated that OSM induces HIF-1α. Research in cancer cells reveals that HIF-1α suppresses cyclin D1 to inhibit proliferation (15). An interesting addition to our story would be to suppress HIF-1α in subconfluent proliferating PAs and examine cyclin D1, as well as cell cycle.

Furthermore, we showed that OSM inhibits adipocyte differentiation. It's generally accepted that inhibition of adipogenesis is associated with reduced insulin sensitivity (16). In this sense, these data further the link between inflammation and adipocyte dysfunction. An important addition to our data would be to examine the effect of OSM overexpression on PPARγ in adipose tissue isolated from mice. These data would give insight into the effects of OSM on adipogenesis in vivo. We revealed that mitotic clonal expansion (MCE) was a critical period for OSM to inhibit differentiation. Addition of OSM after MCE had no effect on the conversion of PAs to adipocytes (ADs). It was a plausible hypothesis that this was due to the loss of OSMRβ over the course of differentiation that our lab and others (4) have seen. However, our data show that the addition of OSM

sustained OSMRβ during differentiation, thereby disproving the hypothesis that the loss of OSMRβ was responsible for the effect of OSM being confined to MCE. In fact, an experiment we performed addressing the effect of MDI components on OSMRβ and PPARγ in late stages of differentiation showed an inverse relationship where PPARγ was expressed the most when OSMRβ was the lowest.

It's been previously document in a published report that there is an association between cyclin D1 and the C/EBP's (17). Recent evidence revealed that cyclin D1 binds to LAP1, the most predominant isoform of C/EBPβ to drive expression of PPARγ for terminal differentiation (18). Our data demonstrated that OSM reduced both cyclin D1 and C/EBPβ and completely inhibited the induction of LAP1 over the course of differentiation. An important experiment to do would be to examine the interaction of cyclin D1 and LAP1 during early differentiation. If cyclin D1 binds to LAP1 and this activates C/EBPβ transcriptional function, this could help explain why OSM inhibits PPARγ. While we found that LAP1 was inhibited by OSM, we demonstrated that the induction of LAP2, which is also a driver of differentiation, was not inhibited. These data suggest that the inhibition of differentiation by OSM could occur by inhibiting protein expression of LAP1 or inhibiting the function of C/EBPβ. Others have shown that the ratio of C/EBPβ isoforms, LAP and LIP, is an important determinant of the fate of differentiation (19). However, we found that regardless of OSM stimulation, the abundance of LIP was never greater than LAP, which rules out the possibility that OSM affects PPARγ by increasing LIP.

Our preliminary data revealed that the loss of HIF-1α prevented the OSM-mediated inhibition of differentiation by restoring PPARγ, aP2 and C/EBPα. Other

research has shown that HIF-1α regulates PPARγ through the regulation of DEC1, which contains functional HREs (20,21). Future examination of the regulation of OSM-induced HIF-1α on DEC1 could provide another link between OSM and the blockade of adipogenesis. Another interesting study to perform would be to examine the effect of overexpression of OSM on PPARγ in adipose tissue from HIF-1α deficient mice. This would help clarify a role for HIF-1α in OSM-mediated inhibition of adipogenesis in vivo. Additionally, we found that HIF-1α suppression partially restored the reduction of cyclin D1 by OSM. While our data showed that OSM accelerated the decay of C/EBPβ over time and completely inhibited LAP1 induction, the loss of HIF-1α partially restored C/EBPβ, as well as LAP1 at d5. Future studies are needed to confirm the relationship between cyclin D1 and C/EBPβ, as well as a functional role for C/EBPβ during late stages of differentiation.

During obesity, adipose tissue undergoes the remodeling process to compensate for its expansion (22). While our studies were limited to the effects of OSM in vitro on proliferation and differentiation, it would be important for future studies to explore the effect of OSM in vivo on other aspects of adipose tissue remodeling, such as alterations in the extracellular matrix (ECM), fibrosis and macrophage infiltration. Collectively, obesity results in adipose tissue dysfunction, which includes impairments in lipid metabolism, autophagy, decreased blood flow, macrophage infiltration and increased pro-inflammatory cytokine secretion (23). Our preliminary studies found that a cytokine (OSM) has the ability to up-regulate a protein named after its induction during hypoxia (HIF-1α). While we found that the addition of OSM and $CoCl_2$ synergistically regulated

HIF-1α, investigations exploring the downstream effects of the combination may be more representative of the magnitude of adaptations that occur during obesity.

In conclusion, our previous chapters have significantly contributed to the knowledge regarding regulation and function of HIF-1α by OSM during normoxia in both PAs and differentiating ADs. Data in this dissertation have led to ample questions for future studies, especially related to the mechanisms by which OSM affects adipose tissue remodeling. Continued research examining the relationship between OSM and HIF-1α will lead to a more comprehensive understanding of how inflammation and hypoxia contribute to adipose tissue remodeling and metabolic adaptation.

Reference List

1. Richards CD. The Enigmatic Cytokine Oncostatin M and Roles in Disease. ISRN Inflamm. 2013;512103.

2. Hermanns HM. Oncostatin M and interleukin-31: Cytokines, receptors, signal transduction and physiology. Cytokine Growth Factor Rev. 2015;26(5):545–58.

3. Vollmer S, Kappler V, Kaczor J, Flügel D, Rolvering C, Kato N, et al. Hypoxia-inducible factor 1α is up-regulated by oncostatin M and participates in oncostatin M signaling. Hepatology. 2009;50(1):253–60.

4. Miyaoka Y, Tanaka M, Naiki T, Miyajima A. Oncostatin M inhibits adipogenesis through the RAS/ERK and STAT5 signaling pathways. J Biol Chem. 2006;281(49):37913–20.

5. Dengler VL, Galbraith MD, Espinosa JM. Transcriptional regulation by hypoxia inducible factors. Crit Rev Biochem Mol Biol. 2014;49(1):1–15.

6. Kietzmann T, Mennerich D, Dimova EY. Hypoxia-Inducible Factors (HIFs) and Phosphorylation: Impact on Stability, Localization, and Transactivity. Front Cell Dev Biol. 2016;4(February):1–14.

7. Trayhurn P. Hypoxia and Adipocyte Physiology: Implications for Adipose Tissue Dysfunction in Obesity. Annu Rev Nutr. 2014;34(1):207–36.

8. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 2003;3(10):721–32.

9. Wang B, Wood IS, Trayhurn P. Hypoxia induces leptin gene expression and secretion in human preadipocytes: Differential effects of hypoxia on adipokine expression by preadipocytes. J Endocrinol. 2008;198(1):127–34.

10. Wood IS, Hunter L, Trayhurn P. Expression of Class III facilitative glucose transporter genes (GLUT-10 and GLUT-12) in mouse and human adipose tissues. Biochem Biophys Res Commun. 2003;308:43–9.

11. Wood IS, De Heredia FP, Wang B, Trayhurn P. Cellular hypoxia and adipose tissue dysfunction in obesity. Proc Nutr Soc. 2009;68(4):370–7.

12. Wood IS, Stezhka T, Trayhurn P. Modulation of adipokine production , glucose uptake and lactate release in human adipocytes by small changes in oxygen tension. Eur J Physiol. 2011;469–77.

13. Wang B, Wood IS, Trayhurn P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. Pflugers Arch Eur J Physiol. 2007;455(3):479–92.

14. Elks CM, Zha P, Grant RW, Hang H, Bailey JL, Burk DH, et al. Loss of Oncostatin M Signaling in Adipocytes Induces Insulin Resistance and Adipose Tissue Inflammation in Vivo*. J Biol Chem. 2016;291(33):17066–76.

15. Wen W, Ding J, Sun W, Wu K, Ning B, Gong W, et al. Suppression of Cyclin D1 by Hypoxia-Inducible Factor-1 via Direct Mechanism Inhibits the Proliferation and 5-Fluorouracil–Induced Apoptosis of A549 Cells. Cancer Res. 2010;70(5):2010–20.

16. Sarjeant K, Stephens JM. Adipogenesis. Cold Spring Harb Perspect Biol. 2012;4(9):1–19.

17. Dean RG, Hausman GJ, Kim HS, Hausman DB, Martin RJ. The Expression of Cyclin D1 during Adipogenesis in Pig Primary Stromal-Vascular Cultures. Obes Res. 2008;9(9):572–8.

18. Liu Q, Boudot A, Ni J, Hennessey T, Beauparlant SL, Rajabi HN, et al. Cyclin D1 and C/EBPβ LAP1 Operate in a Common Pathway To Promote Mammary Epithelial Cell Differentiation. Mol Cell Biol. 2014;34(16):3168–79.

19. Yeh WC, Cao Z, Classon M, McKnight SL. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev. 1995;9(2):168–81.

20. Yun Z, Maecker HL, Johnson RS, Giaccia AJ. Inhibition of PPAR-gamma2 Gene Expression by the HIF-1-Regulated Gene DEC1/Stra13 : A Mechanism for Regulation of Adipogenesis by Hypoxia. Dev Cell. 2002;2:331–41.

21. Tanimoto K, Miyazaki K, Kato Y, Nishiyama M, Kawamoto T, Honda H. Identification of Functional Hypoxia Response Elements in the Promoter Region of the DEC1 and DEC2 Genes. J Biol Chem. 2002;277(49):47014–21.

22. Sun K, Kusminski CCM, Scherer PEP. Adipose tissue remodeling and obesity. J Clin Invest. 2011;121(6):2094–101.

23. Klöting N, Blüher M. Adipocyte dysfunction, inflammation and metabolic syndrome. Rev Endocr Metab Disord. 2014;15(4):277–87.